

# **VITRIFICATION OF BOVINE OOCYTES**

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University of Saskatchewan  
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By

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## ABSTRACT

The overall objective of this thesis was to investigate the vitrification of bovine cumulus oocyte complexes (COCs) as a tool for conservation of female genetics. Specifically, the first objective was to compare *in vitro* maturation rates (i.e. end point Metaphase II (MII) rate) of bovine COCs following vitrification using two different equilibration times (0 vs 10 min) in vitrification solution 1 (VS1) and two different cryodevices (cryotop vs 0.25 mL straw). The MII rate was higher in the non-vitrified control group than in vitrified groups (61 vs 16%,  $P < 0.001$ ). In the vitrified groups, equilibration time in VS1 [TCM-199 + 7.5% ethylene glycol (EG) + 7.5% dimethyl sulfoxide (DMSO) + 20% calf serum (CS)] did not affect MII rate ( $P = 0.964$ ); however, MII rate was higher in COCs vitrified on cryotops than in straws (23 vs 9%,  $P = 0.007$ ).

The second objective of this thesis was to compare cleavage and subsequent developmental competence of bovine COCs vitrified using different vitrification solutions (fresh vs frozen) and different equilibration times in VS1 (0 vs 5 min). Immature bovine COCs were vitrified on cryotops using vitrification solutions either prepared fresh or frozen/thawed, and two equilibration times in VS1 (0 vs 5 min). Cleavage and blastocyst production rates were higher ( $P < 0.001$ ) in the non-vitrified control group than vitrified groups (cleavage rate 93 vs 42% and blastocyst rate 31 vs 0.4%). The cleavage rate of COCs vitrified in frozen/thawed solutions with 5 min equilibration was higher ( $P = 0.05$ ) than in other treatment groups. However, the blastocyst rate did not differ ( $P = 0.993$ ) among vitrified groups.

We hypothesized that the nuclear stage of bovine COCs at the time of vitrification will affect post-warming *in vitro* maturation (IVM), cleavage and embryo development. Firstly, a preliminary study was conducted to validate our *in vitro* maturation system. The COCs were at germinal vesicle (GV, 89%), germinal vesicle breakdown (GVBD, 47%), metaphase I (MI, 90%), and metaphase II (MII, 84%) stages at 0, 6, 12 and 22 h of IVM respectively. In a subsequent study, bovine COCs were vitrified after 0, 6, 12 and 22 h of IVM by loading on cryotops and plunging in liquid nitrogen. Following 1 min in warming solution (TCM-199 + 17% sucrose + 20% CS), COCs were placed in IVM medium to complete 22 h of IVM and nuclear stages were evaluated using lamin A/C-DAPI staining. The nuclear maturation (MII)

rates of COCs were 23, 23, 35 and 89% ( $P<0.001$ ) in the 0, 6, 12 and 22 h IVM groups, respectively. In the final nuclear stage experiment, cleavage and embryo development was determined after vitrification of COCs after 0, 12 and 22 h of IVM. The cleavage and blastocyst rates were higher ( $P<0.001$ ) in the non-vitrified (control) group than vitrified groups (73 vs 15% and 22 vs 0.3%, respectively). The cleavage rates (14% in 0 h, 17% in 12 h and 14% in 22 h groups;  $P=0.825$ ) and blastocyst rates (0% in 0 and 22 h and 1% in 12 h groups) did not differ. Results from this study indicated that a greater proportion of COCs progressed to MII if vitrification occurred at MI rather than at GV and GVBD stages. However, the nuclear status of vitrified COCs appeared to have no effect on fertilization or subsequent embryo development.

A final set of experiments were designed to determine if the exposure of bovine COCs to cryoprotectant solutions and different warming time intervals affects their ability to become fertilized, cleave and develop into blastocysts. In both experiments, the cleavage and blastocyst rates in the vitrified group were lower ( $P<0.001$ ) than in the non-vitrified control groups VS1 group, and in the VS1 + vitrification solution 2 (VS2; TCM-199 + 15% EG + 15% DMSO + 20% CS) group. However, the cleavage and blastocyst rates did not differ ( $P>0.05$ ) in control, VS1 and VS1+VS2 groups. In the second experiment, there was no difference in cleavage rates between 1 and 5 min intervals in the warming solution.

In conclusion, cryotop can be used as a preferred cryodevice for vitrification of bovine COCs. Vitrification solutions (VS1 and VS2) and the warming solution can be prepared and stored in the freezer ( $-20^{\circ}\text{C}$ ) for convenience. The pre-equilibration of bovine COCs in VS1 had no effect on the nuclear maturation of vitrified/warmed COCs, but 5 min pre-equilibration in VS1 improved cleavage rates. The nuclear stage of COCs at the time of vitrification and cryoprotectant exposure appeared to have no effect on subsequent cleavage. Furthermore, there was no difference in cleavage and blastocyst rates following vitrification and 1 or 5 min warming time intervals. When compared to non-vitrified controls, the vitrification of immature bovine COCs resulted in reduced nuclear maturation, cleavage and embryo development rates. Further studies are needed to elucidate the causes of the poor embryo development in vitrified-warmed bovine COCs at genomic, proteomics and metabolomics levels.

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## **LIST OF ABBREVIATIONS**

µl: Microliter

AI: Artificial insemination

ART: Assisted reproductive technologies

BO: Brackett-Oliphant

BSA: Bovine serum albumin

cAMP: Cyclic adenosine monophosphate

CMCF: Canadian Macromolecular Crystallography Facility

CS: Newborn calf serum

COCs: Cumulus-oocyte complexes

CO<sub>2</sub>: Carbon dioxide

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DPBS: Dulbecco's phosphate buffered saline

EG: Ethylene glycol

FAO: Food and Agriculture Organization

FSH: Follicle stimulating hormone

GnRH: Gonadotropin-releasing hormone

GV: Germinal vesicle

GVBD: Germinal vesicle breakdown

h: Hour

ICSI: Intracytoplasmic sperm injection

INT: Intermediate

IVC: *In vitro* culture

IVF: *In vitro* fertilization

IVM: *In vitro* maturation

LH: Luteinizing hormone

LN<sub>2</sub>: Liquid nitrogen

M: Molar

MAPK: Mitogen-activated protein kinase

MI: Metaphase I

MII: Metaphase II

min: Minute

mL: Milliliter

MPF: Maturation promoting factor

n: Number

N<sub>2</sub>: Nitrogen

OPS: Open pulled straw

O<sub>2</sub>: Oxygen

s: Second

vs: Versus

VS1: Vitrification solution 1

VS2: Vitrification solution 2

## CHAPTER 1: INTRODUCTION

Many domestic breeds of livestock are experiencing a gradual diminishment of genetic diversity, mainly due to the selection of few breeds, or lines within breeds, to meet the market demand. However, it is in the interest of the international community to conserve livestock genetic diversity for food security and as an insurance policy against global climate change, disease threats, social changes and unforeseen catastrophes [1, 2]. Ideally, populations could be preserved as live animals; but this is expensive and unless animals can be used for production, is not likely to succeed [2]. Therefore, *in vitro* conservation strategies are being developed to preserve animal genetic resources for long term storage in gene banks that can be used at a later time to regenerate a particular population [3, 4].

Conservation programs have focused efforts on the cryopreservation of gametes, embryos, somatic cells and testis and ovarian tissue. Although considerable progress has been made in both semen and embryo cryopreservation of several domestic species, oocytes are extremely sensitive to chilling and to date, a standardized procedure for the cryopreservation of mammalian oocytes has not been established. Long term storage of oocytes would allow for the development of ova banks, permitting female genetic material to be stored unfertilized until an appropriate mate is selected. Successful cryopreservation of oocytes would allow for the preservation of genetic material from unexpectedly dead animals and would facilitate many assisted reproductive technologies [5-7].

During cryopreservation, oocytes suffer considerable morphological and functional damage; however, the extents of cryoinjuries are highly variable depending on species, sensitivity of the cell structure to cooling, meiotic stage, lipid content and origin (*in vitro* and *in vivo*) [5]. The parameters such as temperature and the permeability, toxicity and time of exposure to cryoprotectants should be taken into account while freezing oocytes [6].

Although the specific molecular pathways disrupted during cryopreservation are not well understood, organelles critical to maintenance and development are damaged. The major impairments in oocytes incurred during freezing procedures are due to ice crystal formation and osmotic injury [8]. Strategies to minimize cryoinjuries during freezing are based on the utilization of optimal cryoprotectants and cooling-warming rates. Numerous cryoprotectants such as dimethyl sulfoxide (DMSO), glycerol and ethylene glycol (EG) have been utilized in the cryopreservation of mammalian embryos and oocytes [6]. Currently, conventional slow-controlled freezing and vitrification are the two main methods being explored for oocyte cryopreservation. During slow-controlled freezing, controlled cooling rates avoid changes in cell shape and osmotic effects by allowing extra- and intra-cellular fluid exchange; however, this method commonly leads to ice crystallization and cell damage. Slow freezing is less successful in species whose oocytes are sensitive to chilling such as cattle, horses, sheep and pigs, as opposed to species such as mice, cats and humans, whose oocytes are less prone to chilling injury. Furthermore, equipment requirements and exposure to cryoprotectants for extended periods of time are major limitations [9]. Vitrification is an alternative method of cryopreservation in which cells are exposed to a higher concentration of cryoprotectants and frozen at an ultra rapid rate. This method of cryopreservation results in a solid glass like

structure and minimal intra-cellular ice crystal formation due to the viscosity of the highly concentrated cryoprotectant used in vitrification solutions and the rapid freezing rate [5]. At present, vitrification is a popular method for cryopreservation of “hard to freeze” bovine embryos due to its simplicity and success rate; however, vitrification of bovine oocytes is still challenging due to their complex structure and sensitivity to chilling [10, 11].

The objectives of this study were to examine several different approaches to vitrification and examine the survivability of vitrified bovine cumulus oocyte complexes by *in vitro* maturation, fertilization and culture.

## **CHAPTER 2: LITERATURE REVIEW**

### **I. CONSERVATION OF ANIMAL GENETIC RESOURCES**

#### ***2.1 Loss of farm animal genetic resources***

Phenotypic characteristics are often the foundation for dividing animals into species, which have great diversity. The domestication of animals first began centuries ago with the dog and has led to the development of specific breeds defined as having “a common genetic background, physical appearance, and genetic stability” [12].

Phenotypic and genotypic breed diversification has evolved from animals being kept in different environments, resulting in the selection for specific characteristics in various regions. Animals kept in large groups have also contributed to breed diversification, which has led to indirect selection for specific traits such as behavior or disease resistance. Moreover, breed diversity has arisen from animals being selected by humans for specific traits such as meat or milk in cattle, eggs or meat in poultry, and wool, meat or milk in sheep [2].

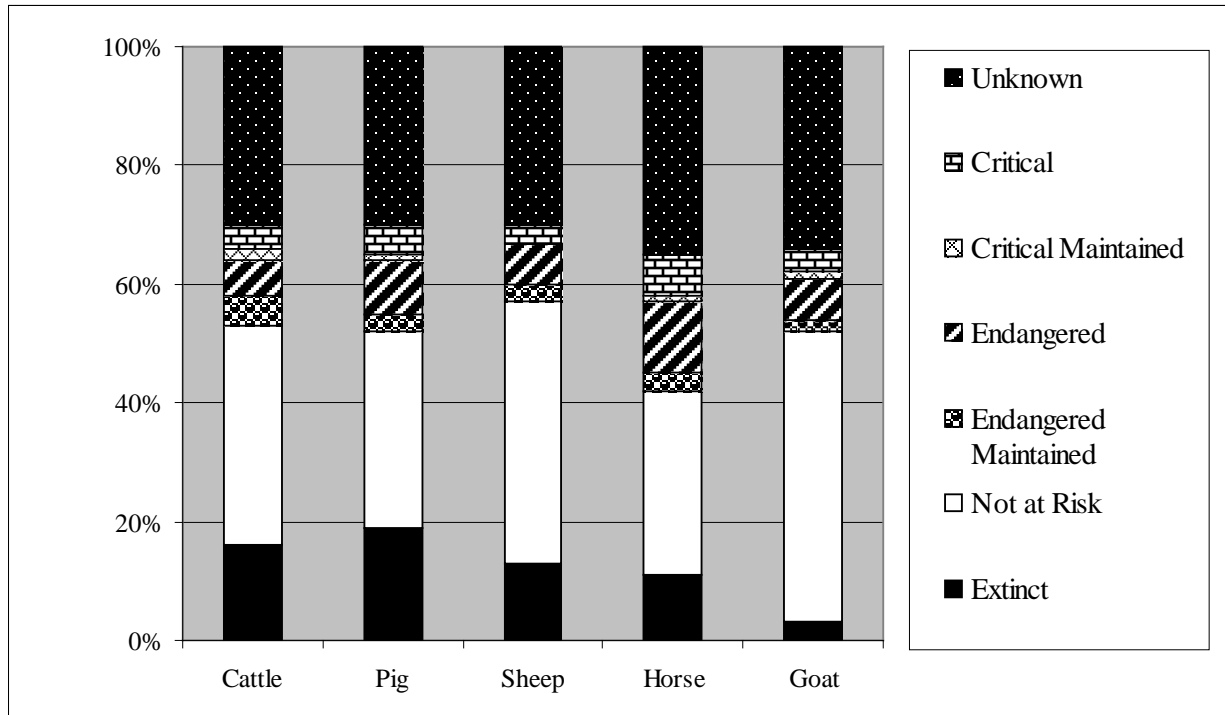
In the last few decades farm animal genetic diversity has rapidly declined, mainly due to changing market demands and intensification of agriculture [1]. Agriculture is moving away from small production systems to large commercial systems and as a result, selection goals and production environments are now very similar throughout the world. Modern reproductive technologies have allowed a large number of progeny to be produced from a single individual



and contemporary transport has enabled the distribution of germplasm around the world rapidly and efficiently. Livestock diversity has also been diminished by many breeding programs carried out by national and international companies, which place intense selection pressure on few breeds [2].

According to the Food and Agriculture Organization (FAO) [2007], approximately 20% of the world's breeds of cattle, goats, pigs, horses and poultry are currently at risk of extinction. At least one livestock breed has become extinct per month over the past several years, resulting in its genetic characteristics being lost forever [13]. Figure 2.1 demonstrates the risk status of several livestock species. Currently over 10% of cattle, pig, sheep and horse breeds are extinct resulting in loss of genetic diversity and potentially useful genes.

In Canada, there are several cattle breeds whose survival and genetic diversity are currently threatened. Table 2.1 demonstrates these breeds according to their risk status [14]. It is imperative to conserve and maintain animal genetic resources to ensure the ability to respond to selection plateaus, consumer demand changes but more importantly biosecurity, environmental and food safety risks by maintaining biodiversity and keeping alternative and potentially useful genes available in the gene pool for present and future use [1, 5, 15].



**Figure 2.1** Percentage of the risk status of the world's cattle, pig, sheep, horse and goat livestock breeds. Critical: the total number of breeding males and females is less than or equal to 100.

Critical maintained: critical populations for which active conservation programmes are in place or populations are maintained by commercial companies or research institutions.

Endangered: the total number of breeding females is greater than 100 and less than or equal to 1000 or the total number of breeding males is less than or equal to 20 and greater than five.

Endangered maintained: endangered populations for which active conservation programmes are in place or populations are maintained by commercial companies or research institutions.

Not at risk: the population has a large amount of breeding males and females and there is no threat of extinction.

Extinct: there are no breeding males or breeding females remaining.

Adapted from the FAO [16].

**Table 2.1** Risk status of cattle breeds. Adapted from Rare Breeds Canada [14].

<b>Status (annual registration of 100% pure female stock)</b>	<b>Breed</b>
Critical (1-25)	Kerry, White Park, Lynch Lineback, Milking Shorthorn
Endangered (26-75)	Belted Galloway, Red Poll, Lincoln Red
Vulnerable (76-250)	Dexter, Canadienne, Galloway, Braunveih, Guernsey
At Risk (251-750)	Highland

## ***2.2 Strategies for farm animal genetic conservation***

Increasing awareness on the reduction of breed diversity has prompted global efforts for conservation of threatened breeds through launching organizations such as Rare Breeds Canada, conferences like the Convention on Biological Diversity and programs such as the Canadian Animal Genetic Resources. Efforts have focused on conservation of diminishing farm animal breeds for several reasons, e.g.; to keep potentially useful genes and gene combinations, to take advantage of heterosis, to overcome selection plateaus, as well as for cultural reasons, research and food security. Maintaining genetic diversity also provides insurance against climate change, disease, changing availability of feedstuffs, social change, selection errors and unforeseen catastrophic events such as Chernobyl where the genetic diversity of many local breeds was threatened [2, 17, 18].

The goals of conservation are to keep genetic variation as gene combinations in a reversible form and to keep specific genes of interest such as the Booroola fecundity gene in sheep [19]. In order to achieve these goals several steps are necessary. First an inventory must be taken to assess and monitor the risk status of a breed in an ongoing basis by examining the number of breeding males and females, the overall breed numbers, the number of sub-populations and the trends in population size. An evaluation of the stocks for phenotype and genotype must be done to determine the genetic distance of one group from another and the choice of breeds for conservation [2].

### ***2.2.1 In situ conservation***

Ideally, populations should be saved as live animals through *in situ* conservation programs; however, this is expensive and unless the breed can be used for production, is not likely to succeed [2]. Hence, *ex situ in vitro* strategies have been developed to cryopreserve animal genetic resources. Genome resource banks create a global gene pool that can be used to manage the exchange of genetic diversity or regenerate a population decades or centuries later [3, 4]. The Convention on Biological Diversity recommended that *ex situ* conservation be complementary to *in situ* conservation for farm animal genetic resources [20].

### ***2.2.2 Ex situ conservation***

One of the major issues surrounding genome banks is the amount and type of material that needs to be stored, which is a function of the intended future use of the material [20]. In order to avoid inbreeding, a gene bank of male and female genetics formed from the largest number of individuals would be ideal [21]. *Ex situ in vitro* conservation programs of livestock genetic resources have focused efforts on cryopreservation of gametes, embryos, somatic cells as well as testis and ovarian tissues, effectively lengthening the genetic lifespan of individuals in a breeding program even after death.

#### ***2.2.2.1 Semen***

Semen is one of the most practical means of storing germplasm due its abundant availability and the ease of application [3, 16]. Stored spermatozoa could be introduced back into existing

populations either immediately or decades or centuries afterwards. Stored frozen-thawed semen from genetically superior males of threatened livestock breeds could be used for artificial insemination (AI) or *in vitro* fertilization (IVF) and has the potential to protect existing diversity and maintain heterozygosity while minimizing the movement of living animals [1, 22]. Breed reconstruction solely from semen is possible through a series of back-cross generations; however, the entire genetics of the original breed will not be recovered, as semen cryopreservation only conserves 50% of genetics [20].

Semen from most mammalian and a few avian species has been successfully frozen in the past several years [16]. However, semen from only highly selected males is cryopreserved, so not all diversity is being stored. Furthermore, the protocols currently used to conserve semen are still sub-optimal and cannot be easily extrapolated across species [23]. Table 2.2 demonstrates the reported average global conception rates in several species following first service AI with frozen-thawed semen [16]. First service conception rates vary drastically between different breeding programs, but on average they are fairly high in cattle, pigs, goats and sheep.

One of the concerns for semen banking in a conservation program is the number of doses that need to be stored. According to the FAO [2007], the number of doses required to regenerate an individual, population or breed is a function of the number of doses needed per parturition, the expected lifetime production of fertile (refounder) females as well as the number of breeding males and females required in the reconstructed population.

**Table 2.2** Average global conception rate in different species following first service artificial insemination (AI) with frozen-thawed semen. Adapted from the FAO [16].

<b>Species</b>	<b>Average global conception rates following first service AI (%)</b>	<b>Number of inseminations</b>
Cattle	50-65	110 million
Pigs	70-80	40 million
Goats	50-80 (intrauterine) or 55-65 (cervical)	120000
Sheep	50-80 (intrauterine) or 55-60 (cervical)	50000
Horses	35-40	5000

The number of semen doses (D) required for breed construction can be calculated as

$$D = d_p * F * n_p$$

where  $d_p$  is the number of doses required per parturition,  $F$  is the total number of females to be inseminated and  $n_p$  is the anticipated number of parturitions per female [24].

It is estimated that in order to conserve a breed from the bovidae family, 1000 semen doses from 25 different males would have to be collected and stored. Furthermore, to reconstruct a horse population of 25 females, the number of doses required would exceed 20000 [20, 25]. In the event that the number of doses available per male is low, the re-establishment of a breed may be more efficient by embryo transfer [16].

#### **2.2.2.2 Embryos**

Embryo cryopreservation allows the conservation of the full genetic complement of both dam and sire and has tremendous opportunities for maintaining heterozygosity and population integrity; but it is a more complex and costly procedure than semen cryopreservation. Moreover, a large number of embryos would be required for complete reconstruction of a population and are unlikely to be available from donor females of endangered breeds [20]. It is anticipated that the collection and storage of 300 unsexed embryos from 90 donors would be required to reconstruct a cattle population; however, finding 90 donor females in an endangered breed would be very challenging and highly unlikely [24].



Embryos of virtually all mammals have been successfully frozen, thawed and transferred to synchronized recipient females in the past [16]. Table 2.3 demonstrates embryo sensitivity towards cryopreservation in different species, developmental stages and origins [5]. Embryos from species such as swine or equine are much more cryo-sensitive compared to bovine or ovine embryos. Currently, the widespread use of embryo cryopreservation is limited to cattle, sheep and goats [26, 27]. Moreover, it is apparent that earlier stage and *in vivo* derived embryos withstand cryopreservation better than later stage and *in vitro* produced embryos. Therefore, the current challenge is to develop a standardized protocol that can be applied to embryos of different species at various developmental stages [5, 16, 28].

#### **2.2.2.3 Oocytes**

Oocytes are the largest cells in the body, have a low surface to volume ratio and are surrounded by a zona pellucida. Immediately adjacent to the oocyte are corona radiata cells that have long cytoplasmic extensions which penetrate the zona pellucida, ending in bulbous swellings closely associated with the oocyte membrane. These processes and gap junctions are important in the metabolic cooperation between the oocyte and surrounding layers of granulosa cells, which form the cumulus oocyte complex (COC) (Figure 2.2) during the growth phase [10]. Gap junctions and cumulus cells play an important role in development of immature oocytes. It has been shown that cyclic adenosine monophosphate (cAMP), which regulates oocyte maturation, originates from cumulus granulosa cells and as cAMP increases in granulosa cells, it also

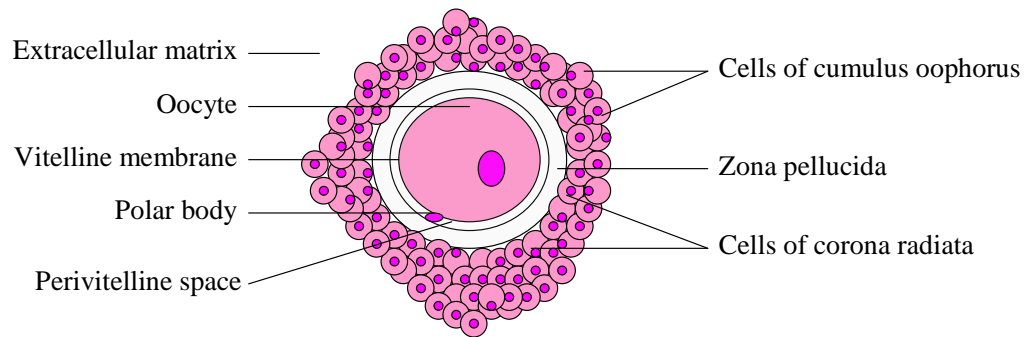
**Table 2.3** Differences of animal oocyte and embryo cryopreservation resistance among species, developmental stages and origin. Adapted from Pereira and Marques, 2008 [5].

	<b>More resistance</b>	<b>Less resistance</b>
Species	Bovine, ovine	Porcine, equine
Developmental stages	Morula, YBL and BL	Hatched BL and oocytes
Origin	<i>In vivo</i> -derived embryos	<i>In vitro</i> -produced embryos, micromanipulated embryos
YBL=young blastocyst; BL=blastocyst		

increases in oocytes [29]. Therefore, osmotic stress on COCs, resulting from the addition and removal of cryoprotectants during cryopreservation techniques may affect oocyte development.

The COCs collected *in vivo* using ultrasound-guided transvaginal retrieval or at slaughter can be frozen for extended periods of time for increased flexibility in their utilization for breeding programs. Oocyte banks would enlarge the gene pool, facilitate several assisted reproductive procedures, salvage female genetics after reproductive failure or unexpected death and avoid controversy surrounding the preservation of embryos [6, 7]. Like semen and embryos, oocyte cryopreservation is beneficial for international exchange of germplasm, as it avoids injury and sanitary risks involved in live animal transportation [5].

The subcellular components of oocytes are extremely temperature and osmotically sensitive. Oocyte cryopreservation is not as established as in semen or embryos, due to the fact that oocytes have a complex subcellular structure and typically have a lower permeability to water and cryoprotectants [23]. The major differences between oocytes and embryos are the plasma membrane, presence of cortical granules and spindle formation at metaphase II (MII) stage of meiosis in oocytes [30]. To date, there has been no consistent oocyte cryopreservation method established in any species. However, there has been significant progress and offspring have reportedly been born from frozen-thawed oocytes in cattle, sheep and horses [31, 32]. In large part, the inability to successfully cryopreserve oocytes can be attributed to the lack of understanding of the basic cryobiological factors that determine survival or death following freezing. During the process of cryopreservation, oocytes suffer considerable morphological and functional damage; although, the extent of cryo-injuries depends on the species and the origin (*in*



**Figure 2.2** Illustration of an ovulated cumulus oocyte complex (COC). The oocyte is encased by a gelatinous matrix and granulosa cells of the cumulus oophorus. Adapted from Schillo [33].

*vivo* or *in vitro* derived). The mechanisms underlying cryoinjuries are yet to be fully understood. Until more insight is gained, improvements in oocyte cryopreservation will be difficult [5].

## **II. OOCYTE CRYOPRESERVATION**

### ***2.3 History***

The cryopreservation of mammalian oocytes has a more recent and far less successful history than spermatozoa and embryos. One of the first investigations into the survival of unfertilized mouse oocytes during cooling and warming was in 1958 [34]. Around 1975, investigation into the fundamental cryobiology of mouse oocytes led to the first report of successful IVF and live offspring from cryopreserved mouse oocytes [35-37]. Although Walter Stiles first suggested vitrification as a cryopreservation strategy in 1930, it was not until the late 1980's that ultra-rapid cooling was used for mouse embryos, which showed promising results when later applied to oocytes [38]. In recent years, there has been increasing interest in the preservation of oocytes from several mammalian species and occasional reports of success have been published in humans [39], bovine [40] and equine [41]. However, there is yet to be a comprehensive understanding of oocyte cryobiology and low fertilization rates need to be resolved before oocyte cryopreservation can be routinely applied.

### ***2.4 Principles***

Cryopreservation involves freezing cells or whole tissues by exposure to sub-zero temperature (-196 °C) in liquid nitrogen (LN<sub>2</sub>) [23]. At such a low temperature, biological activity is effectively stopped and the cells viability and functional state may be preserved for centuries [42]. However, several physical stresses can kill cells at these low temperatures. Intracellular ice formation is one the largest contributors to cell death; therefore, freezing protocols use a combination of dehydration, freezing point depression, supercooling and intracellular vitrification in an attempt to avoid cell damage [43]. However, the removal of excessive water results in cell injury and death through the effect of the highly concentrated intracellular environment on membranes, referred as the “solution effect” [44].

It is important to consider the nature and concentration of the cryoprotectant when cryopreserving germplasm in any cryopreservation protocol. Cryoprotectants such as dimethyl sulfoxide (DMSO), ethylene glycol (EG) or glycerol alone or in combination protect the cells and tissues from freezing damage [5]. Moreover, the cooling rate and freezing method are also important factors to consider in preventing cryoinjuries of cells. Slow freezing, using a controlled freezing curve is commonly used for cell cryopreservation; however, vitrification, which uses an ultra rapid freezing velocity and high concentration of cryoprotectants, is gaining popularity due to its promising success rates in certain species [5].

## ***2.5 Cryoinjuries***

During cryopreservation, the extent of injury incurred in cells largely depends on the size and shape of the cell, the permeability of the cell membranes and the quality (grade) of the cell.

However, these factors vary between species, developmental stage and origin [45]. Although offspring have been born using frozen-thawed oocytes from various species, the ability to support embryo development following cryopreservation procedures is low. This may be attributed to the susceptibility of oocytes to damage during cooling and/or freezing and subsequent thawing because of their complex structure. Unfertilized mammalian oocytes are much larger than the blastomeres of an early embryo and therefore have a small surface to volume ratio [30]. This makes dehydration and penetration of cryoprotectants difficult to achieve, which contributes to the difficulty in cryopreservation. Moreover, the plasma membranes of oocytes differ drastically from those of embryos. Following fertilization, there is a rise in intracellular free calcium, which modifies the ionic strength and membrane potential of the plasma membrane [46]. During embryo development, the submembranous polymerized filamentous actin concentration increases and its conformation changes. This facilitates the permeation of water and cryoprotectants that promotes dehydration and reduces intracellular ice crystal formation during cryopreservation techniques. Additionally, the greater strength of embryos' cell membrane increases the osmotic tolerance during thawing and allows embryos to withstand freezing and thawing better than oocytes [30].

Freezing immature, *in vitro* matured or ovulated oocytes often results in morphological and functional damage. Although the specific molecular pathways disrupted during freezing are not well understood, many ultrastructural elements that are critical to maintenance and development are damaged [47]. Post-thawed oocytes often exhibit zona pellucida or cytoplasmic membrane fractures. Cooling oocytes from approximately 37 °C to 20 °C or below can result in various cytoskeletal and chromosomal modifications but in some cases oocytes have the surprising

ability to fully or partially repair themselves [10, 45]. The major adverse effects of freezing procedures are due to ice crystal formation, osmotic injury, toxic effects of cryoprotectants, concentrated intracellular electrolytes, chilling injury, zona fracture and alterations of intracellular organelles, cytoskeleton and cell to cell contacts [8, 48]. Strategies to overcome these issues involve reducing container volumes, increasing the thermal gradient, altering the cell surface to volume ratio and the addition of substances such as antifreeze proteins, sugars or antioxidants that stabilize the membrane during cooling and increase cryotolerance [5, 6].

The developmental stage of oocytes at the time of cryopreservation is thought to affect the cell's cryobiological properties. Oocytes collected from slaughterhouse-derived ovaries are at the germinal vesicle (GV) stage, at which time the genetic material is contained within the nucleus. The oocyte nucleus is comprised of a nuclear envelope as well as nuclear material such as chromatin and the nucleolus. Reports have shown that freezing immature oocytes is ideal as there is no meiotic spindle present and the genetic material is confined within the nucleus; however, immature oocytes are thought to be more sensitive to anisotonic stress and have lower cell membrane stability than MII stage oocytes [6, 49-51]. Cooling immature oocytes below 4 °C reduces the formation of normal meiotic spindles and fertilization. Additionally, it has been hypothesized that immature oocyte cryopreservation may compromise the structural integrity of the nuclear envelope, subsequently influencing deoxyribonucleic acid (DNA) replication, transcription and cell function; however, the nucleus does have the ability to reassemble following warming [52, 53]. Although a study using time lapse photography on vitrified/warmed oocytes observed that the number of nucleoli returned to a normal state following warming and rehydration, in some cases nucleolar bodies were observed in the



cytoplasm. However, whether intracellular nucleoli localization influences subsequent function and development is still unknown [52].

The success of immature oocyte cryopreservation largely depends on the ability to preserve the structural and functional integrity of the entire COC. Damage to the cumulus cells surrounding the oocyte is also thought to affect the success of immature oocyte cryopreservation. The gap junctions between oocytes and cumulus cells play an important role in the maturation process by providing nutritive substances that have a supportive role during IVF [10]. It has been demonstrated that GV stage oocytes stripped of cumulus cells exhibit deficient nuclear and cytoplasmic maturation [49, 54, 55]. However, cumulus cells can also be an obstacle to the penetration of cryoprotectants [48].

As oocytes mature to the MII stage of maturation, changes occur in the plasma membrane, which affect the permeability of water and cryoprotectants. Once oocytes resume the first meiotic division, the nuclear envelope is disintegrated, allowing the nuclear material to mix into the cytoplasm [52]. There are modifications in nucleolar organization and the distribution of organelles, such as the mitochondria, cytoskeleton and cortical granules, which may also affect the outcome of a cryopreservation technique [56]. Additionally, cumulus cells surrounding the oocyte are expanded, microfilaments of actin are involved in cell shape modifications and movements, and microtubules form the spindle apparatus [10]. Successful fertilization is highly dependent on the maintenance of the structural and functional integrity of COC components. Exposing mature oocytes to cryoprotectants and low temperatures can result in damage to the meiotic spindle, actin filaments, chromosomal dispersal and microtubule depolymerization [10,

57]. Abnormalities of the spindle have been shown to be directly correlated with loss of fertilization and developmental abilities as they are crucial for completion of meiosis, second polar body formation, migration of the pronuclei and formation of the first mitotic spindle [58]

The microtubule, which is a component of the spindle, is a cylindrical bundle, comprised of 13 protofilaments (a heterodimer consisting of  $\alpha$  and  $\beta$ -tubulin). Microtubules begin from microtubular organizing centers at both poles and anchor chromosomes at the kinetochores [30]. The chromosomes align at the equatorial plane of the meiotic spindles. A recent study in porcine oocytes found that paclitaxel treatment improved the normality of microtubules by strengthening the bond between  $\alpha$  and  $\beta$ -tubulin and improved the developmental ability of vitrification of MII oocytes [57, 59].

Regardless of the nuclear stage oocytes are cryopreserved at, DNA may be damaged during cryopreservation [10]. Cryopreserved oocytes have altered distribution of cortical granules, increased polyspermy and zona hardening by premature cortical granule release, which deters the entry of sperm and thus fertilization [5, 59-62]. The use of intracytoplasmic sperm injection (ICSI) has been suggested to overcome the effects of zona hardening. In 1995, a study observed that using ICSI for frozen thawed oocytes resulted in higher fertilization rates than IVF; however, the use of ICSI over IVF requires further investigation [30, 63].

## ***2.6 Criteria to assess the quality of frozen-thawed oocytes***

There are several different methods used to test the viability and extent of chilling injuries of oocytes following cryopreservation. Typically, the primary criteria used to assess post thaw viability of oocytes is the presence/absence of degeneration, cytoplasmic abnormalities (characterized by extensive vasculisation) and zona pellucida fractures [9]. The membrane damage can be evaluated using probes to indicate the integrity of the plasma membrane [6]. Recent studies in humans have examined the meiotic spindle using a polarized microscope apparatus, which allows the visualization of the polymerization of the meiotic spindle following warming. However, this technique is difficult in domestic animals due to their high cytoplasmic lipid content, which hinders spindle examination. Therefore, the oocytes of domestic animals are typically examined through invasive methods such as fluorescence microscopy and biochemical or molecular analyses [9].

Current research is focusing on the development of new oocyte quality and cryotolerance evaluation markers. For example, if the gene expression of the cumulus cells could be correlated with oocyte developmental rates, gene expression could be used as a marker for oocyte quality before freezing, helping to select the oocytes most suitable for cryopreservation [64]. Moreover, determining the volumetric response of matured oocytes to changes in osmolarity during preparation for cooling would be another non-invasive response to oocyte evaluation. This may assist in decreasing the toxic and osmotic effects of cryoprotectants on oocytes. Measuring the volumetric responses to increasing cryoprotectants concentrations should permit a precise estimate of the ideal timing and concentration of cryoprotectants exposure [9]. Additionally, normal vs abnormal gene expression post-thaw could be used to determine which oocytes should be inseminated. Although the viability of frozen-thawed oocytes has been tested using IVF and

ICSI, the best way to evaluate the capacity of embryos derived from cryopreserved oocytes is to produce viable offspring [65]. Currently there have been live offspring born from cryopreserved oocytes in humans [66] and cattle [67].

## ***2.7 Freezing Procedures***

The two main methods studied for cryopreservation of oocytes are slow freezing and vitrification. With the exception of cryoprotectant concentration and cooling, these two methods differ only slightly with regards to storage, warming and rehydration [45]. Table 2.4 compares the two main methods for oocyte and embryo cryopreservation [5].

Conventional slow freezing was introduced first and is currently the gold standard for cryopreservation of embryos. This method typically involves the use of a single cryoprotectant in low concentrations (approximately 1 to 2 M) to minimize chemical and osmotic toxicity and attempts to maintain a balance between the various factors that influence cell damage [5]. During the controlled cooling rate, solution is exchanged between the extracellular and intracellular fluids without serious osmotic effects [45]. However, during slow cooling, extracellular water precipitates as ice, resulting in ice crystal formation. Slow freezing gives acceptable results for oocytes of species that are not sensitive to chilling such as the cat [68, 69], human [39] and mouse [70]. However, bovine and porcine oocytes are more sensitive to chilling and yields poor results following slow cooling [6].

Many plant and animal species located in Polar Regions, where temperatures can fluctuate between -50 °C and +20 °C, survive winter by dehydrating and remaining in a completely vitreous state. Therefore, the pursuit of vitrification in the laboratory can be reassured by its success in nature. The physical definition of vitrification is the glass like solidification of solutions at low temperatures, without the formation of intracellular ice crystals.

During this method of cryopreservation, ice crystal formation is prevented due to the viscosity of the highly concentrated combination of cryoprotectants (approximately 7 to 8 M) used in vitrification solutions that makes water solidify but not expand [48]. Cells undergoing vitrification are frozen at an extremely rapid cooling rate and often undergo fewer physiological detrimental effects compared with slow freezing [11]. Although, some transitional and very short freezing of the solutions can occur during warming following vitrification, this is generally harmless to the oocyte. The disadvantage of using such high concentrations of cryoprotectants is the toxicity to the cells. As a result, oocytes can only be exposed to a minimal volume of vitrification solution for a very short time, i.e., <1 min [5]. Extensive research in the past 20 years has resulted in new approaches which have created an acceptable balance between the positive and negative effects of vitrification. The ultra-rapid cooling rates have allowed decreased cryoprotectant concentrations and made vitrification an extremely competitive alternative to conventional slow freezing [45]. Presently, vitrification is a popular method for cryopreservation of many different cell types, tissues and organs; however, the extent of cryoinjuries and developmental rates are highly variable depending on the species [5, 10].

**Table 2.4** Oocyte and embryo cryopreservation methods. Adapted from Pereira and Marques, 2008 [5].

<b>Freezing procedures</b>	
<b>Conventional slow-freezing method</b>	<b>Vitrification</b>
1. Standard 0.25 mL straws	1. Several devices for loading embryos and oocytes (conventional straws, open pulled straw, cryoloop, cryoleaf, cryotop)
2. Low cryoprotectant concentration	2. High cryoprotectant concentration/ reduced volume and time within vitrification solution
3. Seeding at -5 to -7 °C, controlled slow cooling (0.1 to 1 °C/min)	3. Ultra-rapid cooling rates (-2500 °C/min or 20000 °C/min using open pulled straw and cryoloop)
4. Plunging at -30 to -70 °C and storage in liquid nitrogen (-196 °C)	4. Plunging into liquid nitrogen (-196 °C)

Despite the fact that slow freezing is the most widely used cryopreservation technique, vitrification is a viable and promising alternative that is becoming increasingly attractive to the commercial sector. Many reports comparing conventional embryo slow freezing and vitrification have reported either equal or improved *in vitro* or *in vivo* survival rates after vitrification [71, 72]. Vitrification of oocytes and embryos has been tested in several species with good results, does not require costly coolers or special skill and can be performed fairly quickly [48, 73-75]. It has been suggested that with time, conventional slow freezing will be replaced entirely by vitrification techniques [45].

## **2.8 Cryoprotectants**

Cryopreservation strategies are based on two main principles: cryoprotectants and cooling-warming rates [45]. Because water is not very viscous, it can only be vitrified by extremely rapid cooling of a small sample or using high concentrations of cryoprotectants [44]. Cryoprotectants such as glycerol, DMSO and EG are small molecules that penetrate cells and limit the amount of intracellular and extracellular water that converts into ice during cooling. However, cells do have a biological limit to tolerate the concentration of cryoprotectants. It is imperative to maximize the cooling rate while minimizing the concentration of cryoprotectants during vitrification [44].

Cryoprotective solutions are typically prepared in buffered media (e.g. TCM-199) with a stable pH between 7.2 and 7.4 [76]. Cryoprotectants are compounds used to achieve cellular dehydration and avoid intracellular ice crystal formation upon freezing. Typically, combinations

of permeating and non-permeating cryoprotectants are used. Low molecular weight permeating cryoprotectants such as glycerol, EG and DMSO enter the cell, form hydrogen bonds with intracellular water molecules and lower the freezing temperature, preventing crystallization. Low molecular weight non-permeating cryoprotectants such as sucrose, glucose, trehalose and fructose remain extracellular and draw free water out of the cell by osmosis, resulting in intracellular dehydration [5]. The addition of sugars to an EG based media can strongly influence the vitrification properties of the solution and assist in stabilizing membrane structures [77]. Disaccharides act as osmotic buffers to reduce osmotic shock and the toxicity of EG by decreasing the concentration required to achieve successful cryopreservation [44].

High molecular weight non-permeating polymers or macromolecules are commonly used to reduce the amount of intracellular cryoprotectants necessary for vitrification, reducing the toxicity of the solution. Polymers have been shown to protect against zona pellucida cracking [5]. The majority of solutions used for oocyte vitrification contain a macromolecular component of fetal calf serum (CS) or bovine serum albumin (BSA). Other macromolecules used in vitrification solutions include polyethylene glycol, polyvinylpyrrolidone, Ficoll and polyvinyl alcohol [5].

The addition of non-permeating polymers, a combination of more than one cryoprotectant and their stepwise exposure minimizes the toxic effects of cryoprotectants on cells. Additionally, it has been shown that adding cells to a lower concentration of EG before transferring them to a higher concentration disaccharide mixture can reduce the toxic effect of cryoprotectants [44].



## ***2.9 Cooling Rate***

Although a practical limit to attainable cooling speeds exist, it has been reported that a cooling rate of approximately 2500 °C/min can achieve a vitrified state [76]. However, using a suitable carrier system such as the open pulled straw (OPS), a cooling rate of 20 000 °C/min can be achieved [73]. The main reasons for increasing cooling and warming rates are to avoid ice crystal formation and to decrease the concentration of cryoprotectant solutions [44]. By passing cells through the critical temperature zone (15 to -5 °C) quickly, water is able to move out of the cells and freeze extracellularly [10]. This prevents chilling injury to the intracellular lipid droplets, lipid containing membranes and the cytoskeleton [48].

As cells are immersed in LN<sub>2</sub>, extensive boiling results from the warming of LN<sub>2</sub>. Consequently, evaporation occurs and a vapor coat surrounds the cells and creates an insulating layer that decreases the temperature transfer and cooling rate. Avoiding LN<sub>2</sub> vapor formation through the application of negative pressure with a vacuum can produce a slush state (SN<sub>2</sub>) that decreases the internal temperature and as a result, the cooling rate can be increased [78]. Minimizing the volume surrounding the cell, and establishing direct contact between the cryoprotectant and the LN<sub>2</sub> can also assist in increasing the cooling and warming rates during vitrification of cells [44]. However, techniques based on the direct contact of LN<sub>2</sub> and the medium containing the oocytes may be a source of contamination. These risks can be minimized by using sterile LN<sub>2</sub> for cooling, then wrapping the oocytes in a hermetic container before storage; however, this procedure may be too complex for everyday application [48].

The development of special carriers such as the open-pulled straw [79], microdrops [80], cryoloop [81], flexipet-denuding pipette [82], electron microscopic copper grids [83], hemistraw system [84], small nylon coils [85], nylon mesh [86] and cryotops [87] have helped to achieve higher cooling rates while permitting the use of less toxic and less concentrated solutions [10]. These carriers shorten the time of exposure with the final cryoprotectant before cooling and after warming and the small volume of solution prevents heterogeneous ice formation [48].

### **III. VITRIFICATION OF OOCYTES**

Over the years there has been considerable effort focused on reducing the time of freezing procedures and eliminating the need for programmable cell freezers required during conventional slow freezing. Moreover, equilibrium freezing may not be the most advantageous method to cryopreserve oocytes as they are damaged due to long exposure to temperatures near 0 °C [10]. Vitrification is the alternative method of cryopreservation which uses an ultra rapid cooling rate, eliminating the need for programmable freezing equipment. Furthermore, the vitrification technique uses a high cryoprotectant concentration which avoids water precipitation, preventing intracellular ice crystal formation [5]. In the last several years, almost all advancements in oocyte cryopreservation have been made using vitrification techniques and their use for oocyte and embryo cryopreservation will undoubtedly increase in the future [45].

In 1985, vitrification of mouse embryos emerged as an alternative approach to traditional slow freezing methods [38]. However, the first successful mouse embryo vitrification was

documented in 1993 [88]. Bovine oocytes were able to develop to the blastocyst stage following high cooling rates [89] and pregnancies have been achieved following vitrification of human oocytes [90, 91]. Since this time, there has been a dramatic increase in the number of publications concerning vitrification.

Many variables in the vitrification process exist that can profoundly influence the survival rate of oocytes. The extent of injury and the differences in survival and developmental rates are variable depending on the species, developmental stage and origin [5]. The type and concentration of the cryoprotectant, the temperature of the vitrification solution at the time of cell exposure and the duration of exposure to the final cryoprotectant before plunging in LN<sub>2</sub> are all important factors to consider in order to improve survival rates. The exposure time of oocytes and embryos to cryoprotectants may be shortened, or they are often pre-equilibrated in a vitrification solution containing a lower concentration of permeating cryoprotectants, to avoid any anticipated toxic shock resulting from exposure to higher concentrations of cryoprotectants in final vitrification solution [92-94]. However, it is not yet clear whether a pre-exposure to lower concentrations of vitrification is necessary and if so, what is the optimal time for such exposure. During warming, the main biophysical factor causing cellular disruption is osmotic injury, which can occur during the removal of penetrating cryoprotectants from the cell. The ideal time for oocytes to be suspended in warming solution is still uncertain. If the time is insufficient, osmotic injury can occur, which is especially important given that cryopreserved cells are much more sensitive than non-frozen cells [95].

Moreover, the type of cryodevice used for vitrification influences the cooling rate and size of the vapor coat. Oocytes from humans [96-98], pigs [99], horses [100], sheep [81, 101], cattle [102] and buffalo [103, 104] have developed *in vitro* following cryopreservation using cryotop. Although results are inconsistent, meiotic stage is also thought to contribute to oocyte survivability following cryopreservation [50, 105-109]. The ideal strategy to improve the success of vitrification includes increasing the speed of thermal conduction and decreasing the concentration of cryoprotectants [44].

## **2.10 Humans**

Long term storage of oocytes from women who are late in their reproductive life, have hereditary diseases, abnormal oocytes, wish to delay childbirth until later in their reproductive life or who are in danger of losing ovarian function due to pelvic disease, surgery , chemo- and/or radiotherapy are the most important reasons to cryopreserve human oocytes [5, 56]. Oocyte cryopreservation could assist patients who have a history of difficulty with oocyte collection. Furthermore, women undergoing IVF could freeze excess oocytes and avert repeated ovarian stimulation and oocyte retrieval or use them for oocyte donation. This technique has particular implications for allowing sufficient time for screening donors to prevent disease transmission and in countries that permit the donation of oocytes but not embryos to infertile couples [30, 56]. Although ovarian tissue cryopreservation has shown some success, ovaries are highly sensitive to cytotoxic treatments. Additionally, there is often loss of endocrine and gametogenic function following ovarian tissue cryopreservation. In recent years there has been considerable effort focused on preserving oocytes in order to avoid the ethical, legal and religious problems raised

by freezing embryos [110]. Currently, slow cooling techniques are widely used, although vitrification is increasing in popularity.

Slow cooling using rapid thawing has shown promising improvements in the rate of clinical pregnancies per thawed oocyte and clinical pregnancies per transfer [111]. However, results are still not comparable to those obtained during IVF using non-frozen oocytes. Currently it is unclear why oocytes can survive slow cooling, fertilize and cleave with high rates but later become compromised during implantation. There is undoubtedly damage other than the cytoskeletal apparatus that is compromising the developmental performance [110].

Vitrification has also been used to cryopreserve both immature and mature human oocytes as it avoids cellular lesions caused by ice crystallization that often occurs during slow cooling. However, during the vitrification technique, the sample is in direct contact with LN<sub>2</sub>, increasing the risk for microorganism contamination. Currently cryopreservation of mature, MII human oocytes is challenging. Collection of mature oocytes requires approximately one month's induction, often making it unfeasible in cancer treatments. The alternative is to cryopreserve immature oocytes; however, *in vitro* maturation is required and is currently unreliable [110].

Although results have improved over the last several years, oocyte cryopreservation results are still not comparable with those for frozen embryos and cannot yet be applied to create donor oocyte banks [110]. The use of ICSI has helped conquer premature cortical granule release; however its use needs to be investigated further [30]. The current results are encouraging but the technique still needs to be assessed, particularly with regards to the high concentration of

cryoprotectant required and the contamination risk involved in exposing the samples directly to LN<sub>2</sub> [110]. Improving the cryopreservation technique would solve ethical issues surrounding embryo cryopreservation, egg donation and preservation of fertility for cancer patients and women who wish to extend their child bearing life. Furthermore, human oocyte cryopreservation would solve physiological problems and help maintain excess oocytes generated following superovulation treatment [6].

### **2.11 Cattle**

Although the cryopreservation of bovine oocytes remains a challenge, some of the most encouraging results among domestic animals have been obtained in the bovine, where offspring have been born from immature and mature vitrified oocytes following IVF and culture [112, 113]. Vajta [1998] reported a 25% blastocyst rate on day 8 following vitrification using OPS, thawing, IVF and culture *in vitro*. Although bovine oocytes are much more cryostable than porcine oocytes due to less lipid contents and intracellular lipid droplets and vesicles, they are much more difficult to successfully cryopreserve than bovine embryos [114]. GV stage bovine oocytes have homogenous lipid droplets that show little change following cooling; however their large size and low surface to volume ratio makes it difficult for water and cryoprotectants to move across the plasma membrane [115].

Currently one of the main issues associated with cryopreservation of bovine oocytes is the source. The majority of oocytes are collected from slaughterhouse derived oocytes, which presents a problem because oocytes are derived from follicles of different sizes, and thus

different stages of development, which tolerate freezing differently. The other issue with this source is the fact that subsequent maturation occurs *in vitro* as opposed to *in vivo*, which also influences the freezing tolerance of the oocytes [10].

Good quality bovine oocytes should be used to obtain higher blastocyst formation and healthy offspring following vitrification. It has been shown that oocytes matured *in vivo* demonstrate a significantly higher maturation rate and blastocyst formation than those matured *in vitro* [116, 117]. Oocytes obtained from large growing (subordinate) follicles that are not in the presence of a dominant follicle and have large enough diameters to be considered competent also assists in improving cryopreservation rates of bovine oocytes. Moreover, it has been shown that partially removing cumulus cells several hours after the onset of *in vitro* maturation (IVM) may facilitate improved cryoprotectant penetration while still maintaining supportive and nutritive roles [10].

Although modifying cryopreservation methods to fit the cell type being cryopreserved may be preferable, modifying cells to fit the cryopreservation procedure has also been proposed to improve survival rates [115]. One study examined the possibility of transferring cholesterol to bovine oocytes membranes and found a significant ( $P<0.05$ ) improvement in cleavage rates and development to the 8 cell stage relative to the control (55% vs 41%) [118]. Another study which centrifuged mature bovine oocytes in order to partially remove cytoplasmic lipid droplets prior to vitrification found the frequency of polyspermy was reduced in those surviving freeze-thawing compared to the surviving control oocytes [119]. Modifying the lipid content, removing serum albumin from media and the addition of a membrane stabilizer such as trehalose to the cytoplasm may all be areas worth investigating further [115].

## ***2.12 Other farm animals***

The banking of oocytes from non-human species used for laboratory experiments could be used to preserve specific strains, without the high cost associated with continuous breeding [56]. In farm animals, oocyte cryopreservation allows female's genetic material to be stored unfertilized until a suitable mate is selected [5]. Although offspring have been produced after the transfer of embryos from frozen-thawed oocytes in several species [39, 120], the overall success rate has been low, primarily due to the decreased rate of fertilization following freezing and thawing [76].

Similarly to embryos, porcine oocytes are highly sensitive to low temperatures. Consequently viable piglets have not yet been born from cryopreserved oocytes, despite blastocyst development following cryopreservation of MII stage oocytes [57, 99]. The meiotic spindle of porcine oocytes is extremely sensitive to cryopreservation, resulting in impaired development at meiosis II [30]. However, the main cause of poor survival following vitrification can be attributed to the high intracellular lipid content within porcine oocytes. Porcine GV oocytes contain 2.4-fold more lipid droplets than bovine oocytes [121]. Additionally, dark homogenous lipid droplets as well as grey ones with electron-lucent streaks change morphologically from round to spherical with lucent streaks during cooling [5, 114]. However, it has been demonstrated that the removal of cytoplasmic lipid droplets using delipation increases their freezing tolerance [122]. A recent study combined the removal of cytoplasmic lipid droplets with microtubule stabilization and found vitrified porcine *in vitro* matured MII stage oocytes could develop to the blastocyst stage and maintain the ability to develop into fetuses [57].



Furthermore, the use of cholesterol-loaded cyclodextrin to increase the cholesterol content of oocyte membranes has improved cryotolerance [115].

In horse, immature oocytes undergo significant damage during controlled freezing. Less than 16% reach MII during post thaw maturation, which is less than the 50-80% MII rates typical for non-cryopreserved oocytes [123, 124]. Although MII rates are higher in equine oocytes that have been vitrified (28-46%), approximately 50% of those oocytes that reach MII exhibit spindle abnormalities and poor developmental competence [41, 47, 125]. Much of the damage sustained to equine oocytes is on the mitochondria and gap junctions between the oocyte and surrounding cumulus-corona radiata cells and these interactions are critical to successful maturation and developmental competence [54].

Currently, a limited number of studies have been done on the vitrification of small ruminant oocytes, especially in sheep, where poor developmental rates are obtained following immature [126, 127] and mature [81] oocyte vitrification. Poor ovine oocyte cryopreservation has been attributed to damage to enzymes such as MAP kinase, which is critical to oocyte maturation and subsequent embryo development. A 2007 study revealed that denudation of immature ovine oocytes prior to vitrification increased survival and maturation ability. However, the process also decreased Maturation Promoting Factor (MPF) and Mitogen-Activated Protein Kinase (MAPK) concentrations, which influence meiotic and mitotic cell cycle regulation and developmental competence [49]. The same study also revealed that vitrification alters the functional coupling between oocytes and surrounding cumulus cells which reduces gap junction communication. In addition, there are significant modifications of spindle and chromatin

configurations in vitrified immature ovine oocytes. Current investigations are focusing on the factors influencing the ability of vitrified ovine oocytes to undergo IVF and develop further.

Despite recent advancements, the cryopreservation of oocytes of most mammalian species remains a challenge due to their complex structure. Although there have been an increasing number of publications regarding vitrification of oocytes, more research is required to further elucidate the species-specific mechanisms influencing poor survivability following vitrification. Moreover, additional modifications of oocytes to fit the cryopreservation technique may be necessary, especially in species whose oocytes have extremely high lipid contents.

#### **IV. CONCLUSIONS**

The loss of farm animal genetic diversity is occurring at alarming rates across the globe. Traditions, cultural values and safeguarding diversity for an unpredictable future are all driving forces for genetic conservation, which is a global responsibility. Preserving live animals permits further evolution of breeds; however, in many cases; *in situ* conservation strategies are not practical or adequate. Therefore, establishing genetic resource banks to conserve the current genetic status would provide a crucial interface between *ex situ* and *in situ* conservation strategies.

Cryopreservation of oocytes is a crucial step for the conservation of female genetics. However, despite decades of research, oocyte cryopreservation remains a challenge in virtually all species due to the complex structure of the oocyte. Conventional slow freezing commonly leads to

intracellular ice crystallization and cell damage, and although vitrification of bovine oocytes is being studied increasingly, it is still challenging due to the complex structure of oocytes and sensitivity to chilling. Vitrification is a relatively simple and inexpensive method of cryopreserving oocytes; however, despite tireless efforts over the past 20 years, vitrification has yet to produce convincing results capable of widespread application. It is critical that researchers achieve more consistent results and establish a “universal” protocol that can be applied for the cryopreservation of oocytes at different developmental stages. The goals of my thesis are to establish an efficacious vitrification protocol and increase the ability of vitrified bovine oocytes to cleave and subsequently develop into embryos.

## **V. HYPOTHESES AND OBJECTIVES**

- ***Hypothesis 1:*** Cryotop is a more efficacious packaging system than 0.25 mL straws for vitrification of bovine COCs.
- ***Objective 1:*** Compare nuclear maturation of bovine oocytes *in vitro* following vitrification using two different packaging systems (cryotop vs 0.25 mL straw).
- ***Hypothesis 2:*** Vitrification of immature bovine oocytes with and without equilibration in vitrification solution 1 has similar effects on post-warming nuclear maturation.
- ***Objective 2:*** Compare nuclear maturation of bovine oocytes *in vitro* following vitrification using two different equilibration times (0 vs 10 min).

- **Hypothesis 3:** Vitrication of immature bovine oocytes following an extended equilibration time in vitrication solution 1 has no effect on cleavage and subsequent embryo development.
- **Objective 3:** Compare cleavage and developmental competence of bovine COCs vitricified using different equilibration times (0 vs 5 min).
- **Hypothesis 4:** As compared to frozen-thawed solutions, vitrication of immature bovine oocytes in fresh vitrication solutions result in improved cleavage rates and subsequent embryo development.
- **Objective 4:** Compare cleavage and developmental competence of bovine COCs vitricified using different vitrication solutions (fresh vs frozen).
- **Hypothesis 5:** Immature (GV stage) bovine oocytes will mature *in vitro* within 22 h.
- **Objective 5:** Validate our *in vitro* maturation system.
- **Hypothesis 6:** Nuclear stage of bovine oocytes at the time of vitrication affects their post-warm nuclear maturation *in vitro*, cleavage ability and developmental competence.

- **Objective 6:** Evaluate nuclear maturation, cleavage and subsequent embryo development following vitrification of bovine oocytes at different nuclear stages.
- **Hypothesis 7:** Exposure of bovine COCs to cryoprotectant solutions affects their ability to become fertilized, cleave and develop into embryos. The higher concentrations of cryoprotectant solutions used in vitrification procedures will have toxic effects on bovine COCs, resulting in poor cleavage and embryonic development.
- **Objective 7:** Determine the role of cryoprotectant solutions in the observed reduced rates of cleavage of vitrified bovine oocytes and subsequent embryo development by studying the effect of exposure of bovine COCs to vitrification solutions and vitrification on cleavage and blastocyst rates.
- **Hypothesis 8:** Warming vitrified COCs for a longer interval will improve the removal of intracellular cryoprotectants resulting in higher cleavage and blastocyst production rates.
- **Objective 8:** Study the effect of time of exposure of bovine COCs to warming solutions (1 vs 5 min) on cleavage and subsequent embryo development.

### **CHAPTER 3: FACTORS AFFECTING NUCLEAR MATURATION, CLEAVAGE, AND SUBSEQUENT DEVELOPMENT OF VITRIFIED BOVINE CUMULUS-OOCYTE COMPLEXES**

#### ***3.1 Abstract***

The objective of this study was to investigate the effect of equilibration time, vitrification solutions and cryodevice on *in vitro* maturation and cleavage of vitrified bovine oocytes, and subsequent embryo development. Experiment 1 was designed to compare the nuclear maturation rate (i.e., to Metaphase II; MII) of immature bovine cumulus oocytes complexes (COCs) vitrified using two equilibration time intervals (0 vs 10 min) in vitrification solution 1 [VS1; TCM-199 + 7.5% ethylene glycol (EG) + 7.5% dimethyl sulfoxide (DMSO) + 20% calf serum (CS)] followed by exposure to vitrification solution 2 (VS2; TCM-199 + 15% EG + 15% DMSO + 17% sucrose + 20% CS) for <1 min and two different cryodevices (cryotop vs 0.25 mL straw). The COCs were warmed for 1 min in warming solution (TCM-199 + 17% sucrose + 20% CS). The MII rate was higher in the non-vitrified control group than in vitrified groups (61 vs 16%,  $P<0.001$ ). In the vitrified groups, equilibration time in VS1 did not affect MII rate ( $P=0.964$ ); however, MII rate was higher in COCs vitrified on cryotops than in straws (23 vs 9%,  $P=0.007$ ). In Experiment 2, immature bovine COCs were vitrified on cryotops using vitrification solutions either prepared fresh or frozen/thawed, and two equilibration times in VS1 (0 vs 5 min). Cleavage and blastocyst production rates were higher ( $P<0.001$ ) in the non-vitrified control group than vitrified groups (cleavage rate 93 vs 42% and blastocyst rate 31 vs 0.4%). Cleavage rate of COCs vitrified in frozen/thawed solutions with 5 min equilibration was higher ( $P=0.05$ )

***\*This study has been accepted for publication in Theriogenology.***

than other treatment groups. However, the blastocyst rate did not differ ( $P=0.993$ ) among vitrified groups. In conclusion, the use of cryotops resulted in higher survival rates than 0.25 mL straws for the vitrification of bovine COCs. Furthermore, 5 min equilibration in VS1 resulted in higher cleavage rates than no equilibration time. Compared with non-vitrified controls, the vitrification procedure per se seemed to damage bovine COCs, resulting in reduced nuclear maturation and embryo development rates. However, cleavage rates were within acceptable limits.

### ***3.2 Introduction***

From conservation of animal genetic resources standpoint, the accessibility of frozen semen is abundant but it ensures only one half of the animal genetics. The preservation of the remaining half, i.e. female genetics, is quite challenging because oocytes are scarcely available and are sensitive to cryopreservation techniques [86]. Developing a reliable method for maintaining the viability of mammalian oocytes after long term preservation is important for the conservation of female genetics.

Conventional slow freezing method for cryopreservation of oocytes and embryos often causes osmotic shock and intracellular ice crystallization, resulting in cell damage [9]. Vitrification is an alternate method of cryopreservation in which cells are exposed to high concentrations of cryoprotectants and frozen with an ultra-rapid cooling rate, resulting in an ice-crystal free solid glass-like structure [5]. Presently, vitrification is a popular method for the cryopreservation of bovine embryos [45] due to its simplicity and success rate; however, limited success has been

reported on vitrification of cumulus oocyte complexes (COCs) due to their complex structure and sensitivity to chilling [10].

During vitrification, cells and tissues are exposed to higher concentrations of cryoprotectants and are frozen at ultra-rapid cooling rate [82]. The sudden exposure to high concentrations of cryoprotectants is toxic to oocytes [81, 87, 102]. Therefore, the exposure time of oocytes and embryos to cryoprotectants is shortened, or they are often pre-equilibrated in a vitrification solution 1, “VS1”, which contains a lower concentration of permeating cryoprotectants than vitrification solution 2, “VS2” [92-94]. However, it is not yet clear whether pre-exposure to a lower concentration of vitrification solution is necessary and if so, what the optimal time for such exposure is. We hypothesized that 1) vitrification of immature bovine oocytes with and without equilibration in vitrification solution 1 has similar effects on post-warming nuclear maturation and 2) vitrification of immature bovine oocytes following an extended equilibration time in vitrification solution 1 has no effect on cleavage and subsequent embryo development.

Several cryodevices have been designed to use the minimum sample volume to achieve ultra-fast cooling rate. These devices include open pulled straw (OPS) [73], cryoloop [128], microdrop [113], flexipet-denuding pipette [84, 129], electron microscope copper grid [89], cryotip [130], nylon mesh [131] and the hemi-straw system [132]. One such promising and recent device is the cryotops, which allows the loading of oocytes or embryos with minimal volume [87, 98]. Oocytes from humans [97, 98], pigs [99], horses [100], sheep [81], cattle [102] and buffalo [103] have been successfully cryopreserved using cryotop. However, cryotop poses a biosecurity threat to the cells, which are exposed directly to liquid nitrogen (LN<sub>2</sub>). Therefore, 0.25 mL



straws could be a better alternative to avoid possible contamination concerns. However, the cooling velocity in straws is not as rapid as in cryotop [133]. Therefore, we hypothesized that cryotop is a more efficacious packaging system than 0.25 mL straws for vitrification of bovine COCs.

Although cryopreservation of bovine oocytes is challenging, some of the most encouraging results among domestic animals have been obtained in the bovine [40, 112, 134], where offspring have been born from vitrified oocytes following *in vitro* fertilization (IVF) and *in vitro* culture (IVC) [135]. In 1998, Vajta [73] reported a 25% blastocyst rate on day 8 following vitrification using the OPS method. Immature, germinal vesicle (GV) stage bovine oocytes have homogenous lipid droplets that shows little change following cooling. However the large size and low surface: volume ratio of oocytes makes it difficult for water and cryoprotectants to move across the plasma membrane [115]. To date, there is no standardized procedure for cryopreservation of bovine oocytes.

Generally, the vitrification solutions (VS1 and VS2) are prepared and stored in a freezer (-20 °C) to avoid batch-to-batch difference and improve convenience. Based on the poor nuclear maturation in vitrified oocytes in Experiment 1, we hypothesized that repeated freezing and thawing may change the vitrifiability of cryoprotectants in vitrification solutions.

The objective of this study was to investigate the effect of cryodevice, and equilibration time on *in vitro* maturation (IVM), cleavage and embryo development of vitrified bovine oocytes. In the first study, the effects of equilibration in VS1 (0 vs 10 min) and cryodevice (cryotop vs 0.25 mL

straw) on the nuclear maturation of vitrified bovine COCs were examined. In the second study, the effects of equilibration in VS1 (0 vs 5 min) and vitrification solutions (fresh vs frozen-warmed) on cleavage and subsequent embryo development of vitrified bovine COCs were examined.

### ***3.3 Materials and methods***

#### ***3.3.1 Chemicals and supplies***

Dulbecco's phosphate buffered saline (DPBS), Ca-Mg free DPBS, newborn calf serum (CS), TCM-199 and MEM non-essential amino acids were purchased from Invitrogen Inc. (Burlington, ON, Canada). Lutropin (LH) and Folltropin (FSH) were supplied by Bioniche Animal Health, Inc (Belleville, ON, Canada). All other chemicals and reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada), unless otherwise stated.

#### ***3.3.2 COCs collection and initial processing***

Bovine ovaries were collected from a slaughterhouse and transported to the laboratory at approximately 25 °C within 6 h. Ovaries were washed in normal saline and extra ovarian tissues were removed. Follicles <8mm in diameter were aspirated using a 18-gauge needle attached to a 5 mL syringe containing approximately 1.0 mL of wash medium consisting of DPBS supplemented with 5% CS (v/v). The aspirated follicular fluid was pooled in 50 mL conical tubes and allowed to settle. The COCs were searched under a stereomicroscope, washed (3x) in

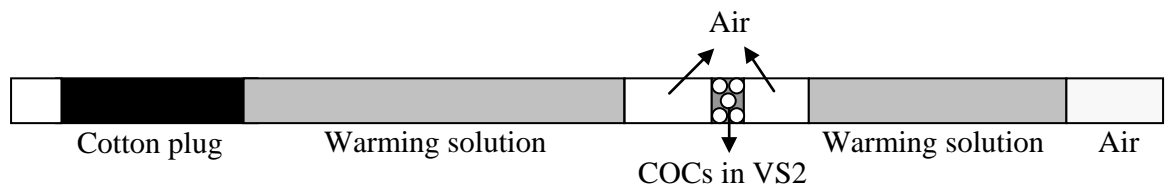
DPBS + 5% CS and COCs with more than three layers of cumulus cells and uniform cytoplasm (Grade One) were selected for further processing [136].

### ***3.3.3 Vitrification and warming procedures***

The COCs were vitrified by equilibrating in VS1 (TCM-199 + 7.5% ethylene glycol (EG; v/v) + 7.5% dimethyl sulfoxide (DMSO; v/v) + 20% CS) for 10 min at 37 °C. After equilibration, COCs were transferred through three 20 µL-drops of VS2 [TCM-199 + 15% EG + 15% DMSO + 20% CS + 17.1% sucrose (w/v)] at 37 °C for a total exposure time of < 1 min. Groups of five equilibrated COCs were loaded in 0.25 mL French straw (IMV, Woodstock, ON, Canada) as shown in Figure 3.1, or placed on cryotop (Kitazato Supply Co., Fujinomiya, Japan) as shown in Figure 3.2, and directly plunged in LN<sub>2</sub>. For warming of COCs, cryotop was individually immersed in 37 °C warming solution consisting of TCM-199 + 20% CS and 17.1% sucrose for 1 min. Straws were individually immersed in 15 mL falcon tube containing 37 °C warming solution for 1 min. The straw contents were emptied in 30 mm Petri dish containing warming solution. After warming, COCs were washed (3x) in DPBS + 5% CS.

### ***3.3.4 In vitro maturation, fertilization and culture***

The COCs were washed (3x) in maturation media (TCM-199 supplemented with 5% CS, 5 µg/mL LH, 0.5 µg/mL FSH and 0.05 µg/mL gentamicin). For IVM, groups of 20 COCs were placed in 100 µL droplets of maturation media, under mineral oil and incubated for 22 h at 38.5 °C, 5% CO<sub>2</sub> in air and high humidity. For IVF, frozen-warmed pooled semen from several bulls



**Figure 3.1** Example of loading 0.25 mL straws for vitrification of cumulus oocyte complexes (COCs). Straw contains columns of warming solution, air, and five COCs in VS2 solution.



**Figure 3.2** Example of cryotop for vitrification of cumulus oocyte complexes (COCs). Cryotop contains a lid, five COCs in VS2 on a thin plastic sheath and a handle.

was washed through a Percoll gradient (45% and 90%) [137]. After washing, sperm were added to Brackett-Oliphant (BO) fertilization media [138] to a final concentration of  $3 \times 10^6/\text{mL}$ . Following 22 h of IVM, groups of 20 oocytes were washed (3x) in BO supplemented with 10% BSA (w/v) and added to 100  $\mu\text{L}$  droplets of sperm in BO, under mineral oil. After 18 h of co-incubation with sperm at 38.5 °C, 5%  $\text{CO}_2$  in air and high humidity, cumulus cells and sperm attached to oocytes were mechanically removed via pipetting. The presumptive zygotes were washed (3x) through IVC medium CR1aa with 5% CS, 2% BME essential amino acids (v/v), 1% MEM nonessential amino acids (v/v), 1% L-Glutamic acid (v/v), 0.3% BSA and 0.05  $\mu\text{g/mL}$  gentamicin, transferred in 100 $\mu\text{L}$  IVC droplets under mineral oil and incubated at 38.5 °C under 5%  $\text{CO}_2$ , 90%  $\text{N}_2$ , 5%  $\text{O}_2$  and high humidity. After 48 h in culture, cleavage rate was recorded and embryo culture was continued in the same drops. Blastocyst rate was recorded on Days 7, 8 and 9 (Day 0= day of IVF).

### **3.3.5 Oocyte staining**

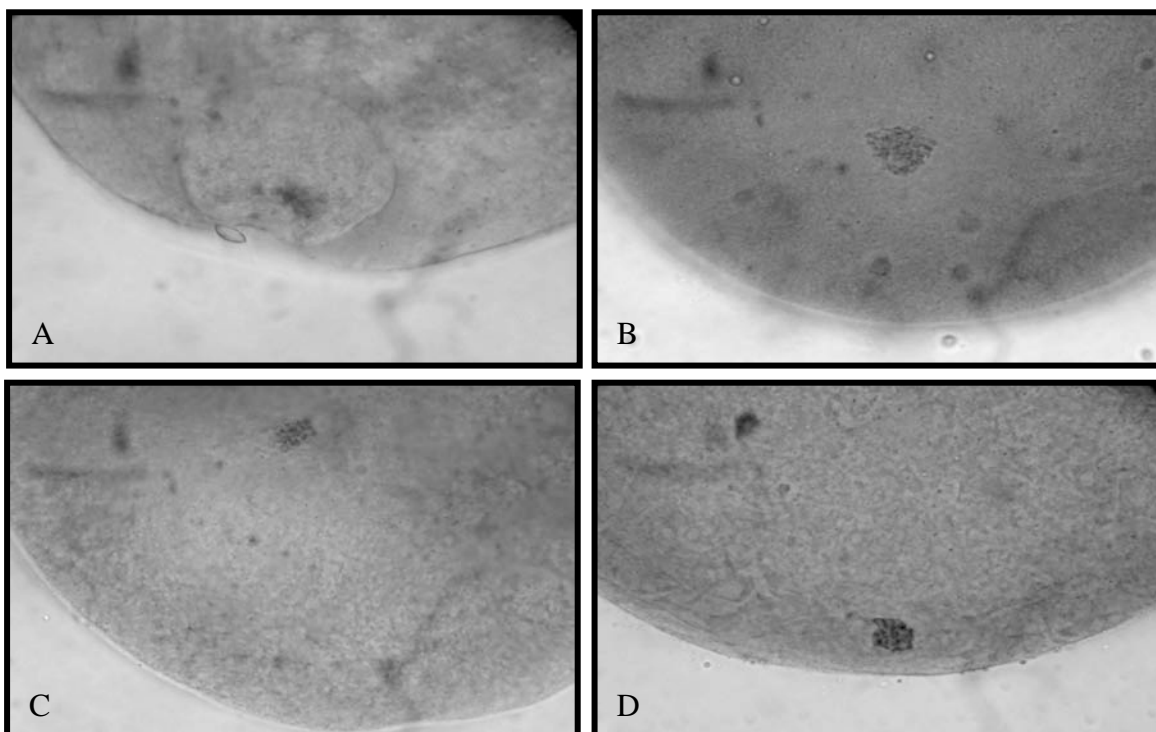
Following 22 h *in vitro* maturation, COCs were completely denuded with 0.3% hyaluronidase in Ca-Mg free DPBS and stained by aceto-orcein method [139] to examine the meiotic status of the oocyte. Briefly, the oocytes were then mounted on microscope glass slide under coverslip (using paraffin-vaseline supports at corners of the coverslip to prevent oocyte rupture) and fixed in ethanol: acetic acid (3:1) for 24 h. The oocytes were stained with 1% orcein (w/v) in 45% acetic acid (v/v) for 20 min and then a minimal amount of differentiate solution (20% glycerol (v/v) and 20% acetic acid) was gently run between the slide and cover slip. The stages of nuclear maturation were determined as previously described [140]. Oocytes were evaluated for the stage

of nuclear maturation as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI) or metaphase II (MII) as shown in Figure 3.3. For data analysis, GV stage oocyte were considered immature, MII oocytes were considered fully mature, and GVBD and MI stages were pooled and designated as intermediate (INT) stages.

### ***3.3.6 Experimental design***

#### ***3.3.6.1 Experiment 1: Effect of equilibration time and cryodevice on nuclear maturation***

The objective of this 2 x 2 factorial design study was to examine the effect of cryodevice and equilibration time on nuclear maturation of vitrified bovine COCs. In one of the treatment groups, COCs were vitrified by equilibrating in VS1 and VS2. In the other treatment group, equilibration in VS1 was skipped and COCs were directly exposed to VS2 for 1 min. Groups of five equilibrated or non- equilibrated COCs were either loaded in 0.25 mL straw (10 min-straw and 0 min-straw groups) by aspiration or placed on cryotop (10 min-cryotop and 0 min-cryotop groups) and directly plunged in LN<sub>2</sub>. Both, non-vitrified (control group) and vitrified COCs were cultured for IVM as described above. All COCs were stained with aceto-orcein method for nuclear evaluation following 22 h culture as mentioned above. This experiment was repeated four times on different dates (replicates).



**Figure 3.3** Classification of bovine oocytes using aceto-orcein staining method: Germinal Vesicle (GV; A), Germinal Vesicle Breakdown (GVBD; B), Metaphase I (MI; C) and Metaphase II (MII; D). Magnification 100 x.



### ***3.3.6.2 Experiment 2: Effect of vitrification solutions and equilibration time on cleavage and embryo development***

The objective of this 2 x 2 factorial design experiment was to examine the effect of vitrification solutions and equilibration time on cleavage and embryo development. The COCs were collected, vitrified, warmed and *in vitro* matured as mentioned above. Collected COCs were randomly divided into two groups. In treatment groups, COCs were exposed to VS1, VS2 and warming solutions either freshly prepared on the day of experiment (fresh solutions group) or frozen at -20 °C (frozen solutions group). Fresh and frozen VS1, VS2 and warming solutions were kept at 37 °C for at least 1 h before use. The COCs in each solutions group were further randomly assigned to equilibration and non-equilibration groups. In equilibration group, COCs were first equilibrated in VS1 for 5 min at 37 °C and then exposed to VS2 at 37 °C (5 min-fresh solutions and 5 min-frozen solutions groups). In non-equilibration group, COCs were directly exposed to VS2 without equilibration in VS1 (0 min-fresh solutions and 0 min-frozen solutions groups). Five of each kind of COCs were placed on each cryotop and plunged in LN<sub>2</sub>. The COCs in fresh solutions were warmed in freshly prepared warming solution and COCs in frozen solutions were warmed in frozen-warmed warming solution, at 37 °C for 1 min. Both, non-vitrified (control) and vitrified COCs were cultured for IVM as described above. Following 22 h of culture, all groups underwent *in vitro* fertilization and culture as mentioned above. This experiment was repeated five times on different dates (replicates).

### ***3.3.7 Statistical analysis***

In Experiment 1, the unclassified and degenerated oocytes were excluded from statistical analysis. In Experiment 2, all the vitrified-warmed oocytes were subjected to IVF and IVC, and included in statistical analysis. Data were analyzed using Proc Glimmix in SAS® Enterprise Guide 4.2 [141]. For both Experiment 1 and 2, two analyses were performed. First analysis compared the non-vitrified control group with combined vitrified groups. Second and final analysis included only the vitrified groups and was performed using a 2x2 factorial randomized complete block design modeling binary distribution (for yes/no response variable). Main effects and interactions were considered statistically different at P-value < 0.05. Replicate number (1 to 4), cryodevice (1=straw, 2=cryotop), equilibration time (1=no equilibration in VS1, 2=10 min equilibration in VS1) and binomial response (MII=1, No MII=2) were recorded for each oocyte. Syntax of SAS program included: Proc glimmix method=quad; class replicate cryodevice equilibration; model MII (event="1") =cryodevice|equilibration /dist=bin link=logit; random intercept/ subject=replicate; run. If the P-value for cryodevice\*equilibration interaction term from Type III sum of squares was < 0.05, then lsmeans cryodevice and equilibration / diff lines ilink or adjust= tukey; was added to the syntax for separation of group means.

### ***3.4 Results***

#### ***3.4.1 Experiment 1: Effect of equilibration time and cryodevice on nuclear maturation***

A total of 64 oocytes were used in non-vitrified control group. After maturation, 10 (16%) oocytes were unclassified and there were no degenerated oocytes in this group. Of the total 260 oocytes in the vitrified groups, 32 (12%) were unclassified and 5 (2%) were degenerated. In

*vitro* maturation rates of bovine oocytes in control and vitrified oocytes are presented in Table 3.1. In the non-vitrified control group, 61% of the oocytes reached the MII stage, compared with 16% in the vitrified groups ( $P<0.0001$ ). In the treatment groups, the nuclear maturation (MII) rate was higher ( $P=0.007$ ) in oocytes vitrified on cryotop than those vitrified in straws (23 vs 9%, respectively). Equilibration time did not affect the outcome ( $P=0.964$ ). Furthermore, a greater number of oocytes remained at the GV stage when vitrified in straws compared with those on cryotop (59 vs 46% respectively;  $P=0.045$ ). Equilibration time had no effect on the proportion of oocytes remaining at GV ( $P=0.497$ ).

#### ***3.4.2 Experiment 2: Effect of vitrification solutions and equilibration time on cleavage and embryo development***

Cleavage and blastocyst rates following vitrification of bovine COCs are presented in Table 3.2. In the control group, cleavage and blastocyst rate were higher ( $P<0.0001$ ) than treatment groups. When the data for vitrified groups were analyzed by factorial design, fresh vs frozen solutions ( $P=0.013$ ) and equilibration time ( $P=0.002$ ) had an effect on cleavage rate. The solution x equilibration time interaction revealed the highest cleavage rate in COCs equilibrated for 5 min in frozen-thawed VS1. The blastocyst rate was low and did not differ due to equilibration times and the preparation method for vitrification solutions ( $P=0.993$ ).

**Table 3.1** Effect of equilibration time and cryodevice on nuclear maturation of vitrified bovine oocytes after 22 h *in vitro* maturation

	Oocytes (n)	Meiotic stage		
		GV <sup>a</sup> n (%)	INT <sup>b</sup> n (%)	MI <sup>c</sup> n (%)
<i>Non-vitrified control versus vitrified</i>				
Non-vitrified control	54	9 (17%)	12 (22%)	33 (61%)
Vitrified	223	118 (53%)	69 (31%)	36 (16%)
P-value		<0.001	0.198	<0.001
<i>Equilibration time in VS1 and cryodevice</i>				
No equilibration				
Straw	65	44 (68%)	15 (23%)	6 (9%)
Cryotop	55	23 (42%)	19 (35%)	13 (24%)
10 min equilibration				
Straw	51	25 (49%)	21 (41%)	5 (10%)
Cryotop	52	26 (50%)	14 (27%)	12 (23%)
<i>P-value</i>				
Equilibration time		0.497	0.411	0.964
Cryodevice		0.045	0.999	0.007
Equilibration time * cryodevice		0.032	0.043	0.901

<sup>a</sup> Germinal vesicle (GV)

<sup>b</sup> Intermediate (INT); germinal vesicle breakdown (GVBD) + metaphase I (MI)

<sup>c</sup> Metaphase II (MII)

### ***3.5 Discussion***

In the present studies, the effect of removing equilibration in VS1 cryoprotectants on oocytes' nuclear maturation, cleavage and embryo development in order to save time during the vitrification procedure was examined. Our interpretation from Experiment 1 demonstrated that there is no beneficial effect of equilibration in VS1 on post-warm nuclear maturation of vitrified bovine COCs. It is plausible that the concentrations of EG and DMSO (7.5% each) in VS1 were still high enough or 10 min exposure time was long enough, to cause chemical toxicity to the COCs. A longer equilibration of bovine COCs in VS1 resulted in lower cleavage and blastocyst rates [81]. The high concentrations of cryoprotectants (EG and DMSO) are toxic to cells. The chemical toxicity of EG and DMSO can be minimized by using combinations of more than one cryoprotectants and a stepwise exposure to cells. The exposure of cells to lower concentrations

**Table 3.2** Effect of vitrification solution preparation and equilibration time on cleavage and blastocyst rate of vitrified bovine oocytes

	Oocytes (n)	Cleavage rate n (%)	Blastocyst rate n (% of total n)
<i>Non-vitrified control versus vitrified</i>			
Non-vitrified control	99	92 (93%)	31 (31%)
Vitrified	482	201 (42%)	2 (0.4%)
P-value		<0.001	<0.001
<i>Solution preparation and equilibration time in VSI</i>			
Fresh			
No equilibration	122	41 (34%)	0 (0%)
5 min equilibration	122	48 (39%)	0 (0%)
Frozen			
No equilibration	128	47 (37%)	0 (0%)
5 min equilibration	110	65 (59%)	2 (2%)
<i>P-value</i>			
Solution preparation		0.013	0.993
Equilibration time		0.002	0.993
Solution preparation * equilibration time		0.079	<0.001

of cryoprotectants (in VS1) before transferring them to high concentrations (in VS2) can potentially reduce the toxic effect of cryoprotectants [44, 93, 94]. The equilibration of oocytes and embryos in VS1, which contains half of the concentration of permeating cryoprotectants of VS2, is generally recommended before vitrification [93, 94, 113]. It is expected to prime oocytes and embryos to minimize any anticipated toxic shock due to high concentration of cryoprotectants in VS2, i.e. 15% EG and DMSO each. Furthermore, equilibration is thought to regulate the permeability of the plasma membrane, which helps in maintaining intercellular connections between the oocyte and cumulus cells, and may assist in avoiding quick osmotic changes [142]. It has been reported that equilibrating embryos results in minimal ultrastructural damage to the plasma membrane [143]. However, based on our results, we concluded that 10 min equilibration of bovine COCs in VS1 may not be essential.

In conventional semen and embryo cryopreservation methods, the French straw is a popular cryodevice (packaging system) as it is inexpensive, and cells and tissues remain sterile. However, vitrification of bovine oocytes in 0.25 mL straws causes a delay in heat loss from the solutions leading to devitrification, i.e. intracellular recrystallization during warming [82]. Recently, considerable progress has been made in increasing cooling and warming rates by developing alternative packaging systems [81]. Cryodevices such as OPS [73], and cryotop [98] have drastically improved the cooling rate by reducing the surface to volume ratio and thus exposing the minuscule vitrification drop directly to LN<sub>2</sub> [84]. In this study, cryotop and 0.25 mL straw as a cryodevice for packaging of bovine COCs for vitrification were compared. From the data, we deduced a higher nuclear maturation in COCs vitrified on cryotop than in 0.25 mL straw. This can be attributed to extremely fast cooling and warming rates achieved using the

cryotop [98]. Cryotop allows COCs to be loaded with minimal VS2 by aspirating the maximum VS2 surrounding COCs. This helped in achieving an ultra-rapid cooling rate, thereby avoiding chilling injury. Additionally, the extremely small volume allowed oocytes to undergo rapid warming rate which helped to avoid devitrification. In 0.25 mL straws, COCs were loaded with a larger volume of VS2 than cryotop, which reduced the cooling rate. Our results are consistent with those previously reported for vitrification of bovine [87] and porcine [99] oocytes in which cryotop yielded higher cleavage and blastocyst rates than OPS. However, ovine oocytes showed the least parthenogenetic activation and higher blastocyst rate when vitrified in cryoloop than cryotop and OPS [81].

In Experiment 2, the ability of vitrified bovine oocytes to achieve normal cleavage following *in vitro* fertilization using fresh or frozen-warmed solution as well as two equilibration times (0 vs 5 min) in VS1 was examined. From our results, we inferred frozen-warmed VS1, VS2 and warming solutions yielded better cleavage rates than fresh solutions, albeit for unknown reason. Moreover, it is unclear whether the cleavage resulted from fertilization or parthenogenesis. The blastocyst rate between fresh and frozen solutions was extremely low and not different. The equilibration of bovine COCs in VS1 for 5 min resulted in a higher cleavage rate than no equilibration. It seems 5 min is an appropriate time for penetration of cryoprotectants with minimum toxicity. The effect of equilibration time or media preparation on blastocyst development was non significant. This is in agreement with a previous report [144].

In conclusion, equilibration of bovine COCs in VS1 is not essential for their *in vitro* nuclear maturation but is important for fertilization process. Cryotop is a better cryodevice (packaging



system) for vitrification of bovine COCs than straws. Furthermore, the present study revealed that vitrification and warming solutions can be prepared in advance and stored below 0 °C with no effect on cleavage or blastocyst rates following vitrification, warming and *in vitro* fertilization, which will make cryopreservation protocols more efficient and convenient. The results of this study can be used to optimize post-warming cleavage and blastocyst rates of vitrified bovine COCs.

## CHAPTER 4: THE EFFECT OF MEIOTIC STAGE OF BOVINE OOCYTES ON THE SURVIVAL OF VITRIFIED CUMULUS OOCYTE COMPLEXES

### 4.1 Abstract

Vitrification is a rapid freezing method in which cells/tissues are frozen in a glass state, bypassing ice crystal formation. However, vitrification of bovine oocytes is challenging due to their complex structure and sensitivity to chilling. The present study aimed to evaluate *in vitro* maturation, cleavage and embryo development of bovine cumulus oocyte complexes (COCs) vitrified at different meiotic stages. In Experiment 1, meiotic progression of oocytes was evaluated at different time intervals during *in vitro* maturation (IVM). Germinal vesicle (GV), GV and germinal vesicle breakdown (GVBD), metaphase I (MI), and metaphase II (MII) oocytes were predominantly found at 0, 6, 12 and 22 h of IVM, respectively ( $n \geq 62$  per group). In Experiment 2, bovine COCs at 0, 6, 12 and 22 h of IVM were exposed to vitrification solution 2 (15% dimethyl sulfoxide + 15% ethylene glycol + 0.5 M sucrose + 20% CS in TCM-199), loaded onto a cryotop device and vitrified by plunging in LN<sub>2</sub>. Following warming, COCs completed 22 h of IVM and nuclear stage was evaluated with lamin A/C-DAPI staining. Upon completion of the 22 h of IVM, 23, 23, 35 and 89% of oocytes from 0, 6, 12 and 22 h groups, respectively were detected at MII ( $P < 0.0001$ ). In Experiment 3, cleavage and embryo development of oocytes vitrified at 0, 12 and 22 h of IVM were evaluated. The cleavage rate did not differ among vitrification group i.e., 14% at 0 h, 17% at 12 h and 14% at 22 h ( $P = 0.825$ ). Cleavage and blastocyst rates were higher ( $P < 0.0001$ ) in the non-vitrified (control) group than in vitrified groups, i.e., 73 vs 15% and 22 vs 0.3%, respectively. In conclusion, vitrification adversely affected the ability of bovine oocytes to undergo *in vitro* maturation to MII stage, *in*

*vitro* fertilization and embryo development. The nuclear stage of bovine oocytes at the time of vitrification had no effect on *in vitro* maturation, cleavage or embryo development.

## **4.2 Introduction**

Many livestock breeds are losing genetic diversity due to the selection of few breeds to meet the market demand. Significant progress has been made in cryopreservation of semen and embryos of several domestic species; however, it is very difficult to cryopreserve cumulus oocyte complexes (COCs). The size of oocytes, sensitivity to chilling, presence of cumulus cells, dynamic nature of subcellular organelles, active biochemical processes and meiosis have made it difficult to develop an efficient cryopreservation procedure [107]. Recently, a vitrification technique that employs an ultra rapid freezing velocity with higher concentrations of cryoprotectants was used to effectively cryopreserve mouse oocytes and has shown some success in other species [98, 104, 105, 107, 145]. Such a protocol for long term preservation of oocytes from domestic animals would be highly beneficial and would allow the development of ova banks permitting the storage of female (unfertilized) genetics until an appropriate sire is selected.

The extent of cryoinjuries to COCs are highly variable depending on species, sensitivity to cooling, lipid contents and origin (*in vitro* and *in vivo*) [5, 6]. Although the results are controversial, meiotic stage is also thought to contribute to oocyte survivability following cryopreservation [50, 105-109]. Preliminary data from our laboratory demonstrated poor maturation rate in vitrified bovine oocytes. Therefore, the objectives of the present study were to determine the maturation kinetics of bovine oocytes in our lab conditions and to evaluate the

effect of meiotic stage of bovine oocytes at the time of vitrification on *in vitro* maturation (IVM), cleavage and embryo development. We hypothesized that 1) immature (GV stage) bovine oocytes will mature *in vitro* within 22 h, 2) nuclear stage of bovine oocytes at the time of vitrification affects their post-warm nuclear maturation *in vitro*, cleavage ability and developmental competence and 3) exposure of bovine COCs to cryoprotectant solutions affects their ability to become fertilized, cleave and develop into embryos. The higher concentrations of cryoprotectant solutions used in vitrification procedures will have toxic effects on bovine COCs, resulting in poor cleavage and embryonic development.

#### ***4.3 Materials and methods***

##### ***4.3.1 Chemicals and supplies***

Dulbecco's phosphate buffered saline (DPBS), Ca-Mg free DPBS, newborn calf serum (CS), TCM-199 and MEM non-essential amino acids were purchased from Invitrogen Inc. (Burlington, ON, Canada). Lutropin (LH) and Folltropin (FSH) were supplied by Bioniche Animal Health, Inc (Belleville, ON, Canada). All other chemicals and reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada), unless otherwise stated.

##### ***4.3.2 COCs collection and initial processing***

Bovine ovaries were collected from a slaughterhouse and transported to the laboratory at approximately 25 °C within 6 h. Ovaries were washed in normal saline and extra ovarian tissues

were removed. Follicles <8mm in diameter were aspirated using a 18-gauge needle attached to a 5 mL syringe containing approximately 1.0 mL of wash medium consisting of DPBS supplemented with 5% CS (v/v). The aspirated follicular fluid was pooled in 50 mL conical tubes and allowed to settle. The COCs were searched under a stereomicroscope and washed (3x) in DPBS + 5% CS. The COCs with more than three layers of cumulus cells and uniform cytoplasm (Grade One) were selected for further processing [136].

#### ***4.3.3 Vitrification and warming procedures***

The COCs were exposed to three 20 µL droplets of vitrification solution 2 [VS2; TCM-199 + 15% ethylene glycol (EG; v/v) + 15% dimethyl sulfoxide (DMSO; v/v) + 20% CS + 17.1% sucrose (w/v)] for less than one min at 37 °C. Five COCs were loaded on a cryotop (Kitazato Supply Co., Fujinomiya, Japan), under stereomicroscope and immediately plunged in liquid nitrogen (LN<sub>2</sub>). The COCs were warmed by immersing the cryotop in a 30 mm petri dish containing warming solution (TCM-199 + 20% CS and 17.1% sucrose) at 37 °C for 1 min. The COCs were then washed (3x) in wash medium at 37 °C.

#### ***4.3.4 In vitro maturation, fertilization and culture***

The COCs were washed (3x) in maturation media (TCM-199 supplemented with 5% CS, 5 µg/mL LH, 0.5 µg/mL FSH and 0.05 µg/mL gentamicin). For IVM, groups of 20 COCs were placed in 100 µL droplets of maturation media, under mineral oil and incubated for 22 h at 38.5 °C, 5% CO<sub>2</sub> in air and high humidity. For *in vitro* fertilization (IVF), frozen-warmed pooled

semen from several bulls was washed through Percoll gradient (45% and 90%) [137]. After washing, sperm were added to Brackett-Oliphant (BO) fertilization media [138] to a final concentration  $3 \times 10^6/\text{mL}$ . Following 22 h of IVM, groups of 20 oocytes were washed (3x) in BO supplemented with 10% BSA (w/v) and added to 100  $\mu\text{L}$  droplets of sperm in BO, under mineral oil. After 18 h of co-incubation with sperm at 38.5 °C, 5%  $\text{CO}_2$  in air and high humidity, cumulus cells and sperm attached to oocytes were mechanically removed via pipetting. The presumptive zygotes were washed (3x) through *in vitro* culture (IVC) medium CR1aa with 5% CS, 2% BME essential amino acids (v/v), 1% MEM nonessential amino acids (v/v), 1% L-Glutamic acid (v/v), 0.3% BSA and 0.05  $\mu\text{g}/\text{mL}$  gentamicin, transferred in 100 $\mu\text{L}$  IVC droplets under mineral oil and incubated at 38.5 °C under 5%  $\text{CO}_2$ , 90%  $\text{N}_2$ , 5%  $\text{O}_2$  and high humidity. After 48 h in culture, cleavage rate was recorded and embryo culture was continued in the same drops. Blastocyst rate was recorded on Days 7, 8 and 9 (Day 0= day of IVF).

#### ***4.3.5 Immunohistochemistry for evaluation of oocyte's nuclear maturation***

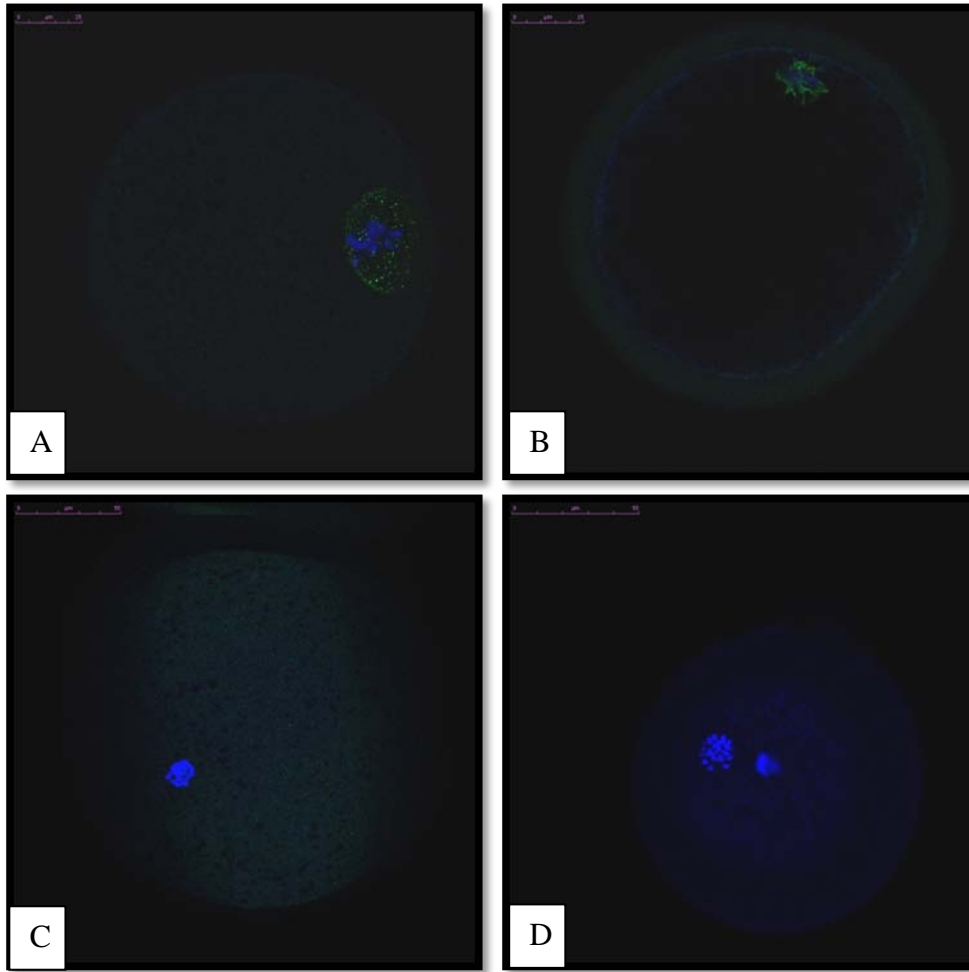
To assess nuclear maturation, oocytes were immunostained using lamin-A/C/DAPI procedure (Anzar et al, unpublished). The COCs were completely denuded by pipetting with 0.3% hyaluronidase in Ca-Mg free Dulbecco's phosphate buffered saline (DPBS) and fixed with 4% paraformaldehyde (w/v) in DPBS. All steps were performed at room temperature and oocytes were washed in DPBS (3x) between steps. Oocytes were permeabilized with 0.5% Triton X-100 (v/v) in DPBS for 30 min followed by 0.05% Tween-20 (v/v) (BIO-RAD, Hercules, CA, USA) in DBPS for 30 min. Oocytes were then placed in a blocking buffer (2% BSA in DPBS) for 60 min. Oocytes were added to mouse anti-lamin A/C (1:300) (Santa Cruz Biotechnology, Santa

Cruz, CA, USA) in DPBS + 2% BSA followed by 1:200 secondary antibody Alexa 488 labeled anti-mouse IgG (Santa Cruz Biotechnology) in DPBS + 2% BSA, each for 60 min. Oocytes were transferred through three 5 µl-drops of Vectashield Mounting Medium containing DAPI (Vector Laboratories Inc., Burlingame, CA, USA), mounted on a microscope glass slide under coverslip (using paraffin-vaseline support at each corner of the coverslip to prevent oocyte rupture) and examined using Zeiss epi-fluorescence microscope for nuclear stage. Nuclear status was classified into one of four stages: germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), and metaphase II (MII), as shown in Figure 4.1. For data analysis, the oocytes at GV and MII stages were considered immature and fully mature, respectively.

#### ***4.3.6 Experimental design***

##### ***4.3.6.1 Experiment 1: Kinetics of nuclear maturation***

The COCs were incubated in maturation medium in groups of 20 for 0, 6, 12 and 22 h in four replicates. Oocyte nuclear maturation was examined at the end of IVM using the lamin A/C-DAPI staining procedure as described above.



**Figure 4.1** Classification of bovine oocytes using lamin A/C DAPI staining method: Germinal Vesicle (GV; A), Germinal Vesicle Breakdown (GVBD; B), Metaphase I (MI; C) and Metaphase II (MII; D).



#### ***4.3.6.2 Experiment 2: Maturation of oocytes vitrified at different nuclear stages***

The COCs were vitrified at 0, 6, 12 and 22 h of IVM, warmed, washed (3x) then placed in 100  $\mu$ L droplets of maturation medium to complete 22 h of IVM. Oocytes vitrified at 22 h were considered as positive controls because they were expected to be mature at the time of vitrification. The COCs that were neither vitrified nor *in vitro* matured (i.e. non-vitrified COCs at 0 h) were considered as control. The nuclear status of oocytes was evaluated using lamin A/C-DAPI staining as described above. This experiment was replicated five times on different dates.

#### ***4.3.6.3 Experiment 3: Developmental competence of oocytes vitrified at different nuclear stages***

The COCs matured for 0, 12 or 22 h were vitrified, warmed, washed (3x) in maturation media, and then returned to 100  $\mu$ L droplets of maturation medium to complete 22 h of IVM. Both the control (non-vitrified) and the vitrified oocytes underwent IVF and subsequent IVC. Cleavage was evaluated on Day 2 (Day 0= day of IVF) and blastocyst rates were determined on Days 7, 8 and 9. This experiment was replicated three times on different dates.

#### ***4.3.7 Statistical analysis***

The level of significance was set at  $P < 0.05$ . Analyses were conducted on each data set with proc glimmix in SAS<sup>®</sup> version 9.2 [141] using binary distribution (for yes/no response variable) and considering replicate as a random factor. In Experiments 1 and 2, the effect of time on

maturation stage (GV, GVBD, MI and MII) was examined. In Experiment 2, 0 h non-vitrified control and 22 h vitrified groups were for comparative purposes and were not included in the second analyses, which compared only 0, 6 and 12 h groups. In Experiment 3, initial analysis compared the cleavage and blastocyst rates between non-vitrified (control) and vitrified groups (combined data from 0, 12 and 22 hr vitrified groups). A second analysis compared the effect of vitrification at 0, 12 and 22 h after initiation of IVM on maturation, cleavage and blastocyst rates within vitrified groups (i.e., excluding non-vitrified control).

## **4.4 Results**

### **4.4.1 Experiment 1: Kinetics of nuclear maturation**

Data on oocytes at GV, GVBD, MI and MII during 22 h of maturation are presented in Figure 4.1. The frequency of GV was greater ( $P<0.05$ ) at 0 h (89%) than the other time periods examined. At 6 h, 47% of oocytes were GV, whereas 44% were GVBD. The frequency of MI (90%) and MII (84%) oocytes was higher ( $P<0.05$ ) at 12 and 22 h respectively, during *in vitro* maturation, than other time periods.

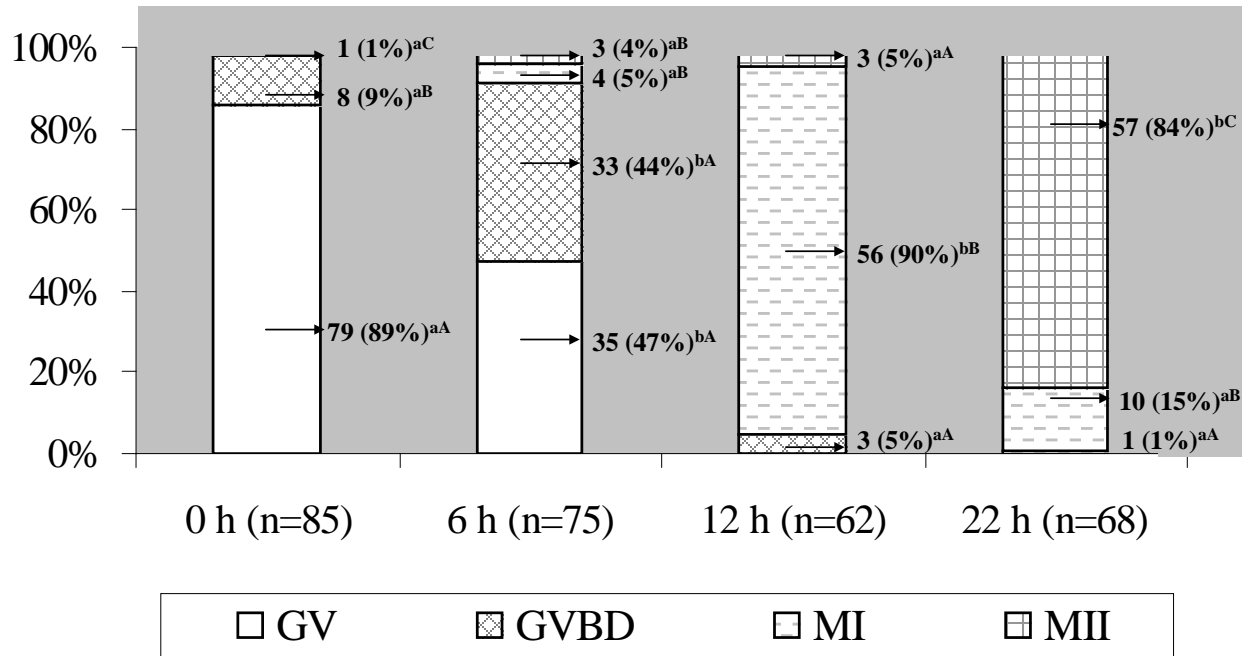
### **4.4.2 Experiment 2: Maturation of oocytes vitrified at different nuclear stages**

Data on maturation rates following vitrification at different times in IVM are shown in Table 4.1. In the non-vitrified control group, 90% of oocytes were immature GV at 0 h compared to less than 15% GV oocytes at 0 h in all other groups ( $P<0.001$ ). Higher number of oocytes were

detected at MII stage in the group vitrified at 22 h (i.e., positive control; 89%, 109/123) than those vitrified at 0, 6 and 12 h ( $P<0.05$ ). When vitrification was performed at or before MI (data combined for 0, 6 and 12 h groups), 25% (97/359) of COCs reached MII. Maturation (MII) of oocytes vitrified at 0, 6 and 12 h of IVM did not differ ( $P>0.05$ ). However, a chi-square comparison detected a difference ( $P=0.027$ ) in MII rate between 12 h group (35%; 42/121) compared to 0 h and 6 h groups combined (23%; 55/238). Compared to 6 and 12 h, a considerable proportion of oocytes vitrified at 0 h (14%) remained at GV stage following 22 h *in vitro* maturation.

#### ***4.4.3 Experiment 3: Developmental competence of oocytes vitrified at different nuclear stages***

Data on cleavage and embryo development of non vitrified controls and vitrified oocytes are shown in Table 4.2. Cleavage and blastocyst rates were higher in non-vitrified (control) COCs (73 and 22%, respectively) than vitrified COCs (0, 12 and 22 h groups combined; 15%; and 0.3%, respectively). Within vitrification groups, cleavage rates did not differ. No blastocyst developed in the 0 and 22 h groups; however, the blastocyst rate as a percentage of oocytes cleaved was 6% in the 12 h group. Due to low numbers, no statistical comparisons were done on blastocyst rates between treatment groups.



**Figure 4.2** Classification of oocyte meiotic stage (GV: germinal vesicle, GVBD: germinal vesicle breakdown, MI: metaphase I and MII: metaphase II) by lamin A/C-DAPI staining procedure at 0, 6, 12 and 22 h of *in vitro* maturation

\*Different superscripts (a, b, c) denote difference within nuclear stage

\*\*Different superscripts (A, B, C) indicate difference within *in vitro* maturation time (0, 6, 12 and 22 h)

**Table 4.1** Ability of bovine oocytes vitrified at different meiotic stages to complete maturation

Oocytes (n)		Meiotic stage			
		GV <sup>a</sup> n (%)	GVBD <sup>b</sup> n (%)	MI <sup>c</sup> n (%)	MII <sup>d</sup> n (%)
0 h (non-vitrified control)	113	102 (90%)	6 (5%)	3 (3%)	2 (2%)
22 h (positive control)	123	1 (1%)	0 (0%)	13 (11%)	109 (89%)
<i>IVM time at vitrification*</i>					
0 h	127	18 (14%)	54 (43%)	26 (20%)	29 (23%)
6 h	111	3 (3%)	30 (27%)	52 (47%)	26 (23%)
12 h	121	0 (0%)	2 (2%)	77 (64%)	42 (35%)
<i>P-value</i>		0.013	<0.001	<0.001	0.068

<sup>a</sup> Germinal vesicle (GV)<sup>b</sup> Germinal vesicle breakdown (GVBD)<sup>c</sup> Metaphase I (MI)<sup>d</sup> Metaphase II (MII)

\*The 22 h of IVM was completed following vitrification and warming

**Table 4.2** Cleavage and developmental rates of bovine oocytes vitrified at different meiotic stages

	Oocytes (n)	Cleavage rate n (%)	Blastocyst rate n (% of total)	Blastocyst rate (% of cleaved)
<i>Non-vitrified control vs. vitrified</i>				
Non-vitrified control	103	75 (73%)	23 (22%)	(31%)
Vitrified	286	43 (15%)	1 (0.3%)	(2%)
P-value		<0.001	<0.001	<0.001
<i>IVM time at vitrification</i>				
0 h	84	12 (14%)	0 (0%)	(0%)
12 h	95	16 (17%)	1 (1%)	(6%)
22 h	107	15 (14%)	0 (0%)	(0%)
P-value		0.825	-	-

#### 4.5 Discussion

In the present study, *in vitro* maturation, cleavage and embryo development following vitrification of bovine oocytes at different nuclear stages were examined. Only 25% of COCs reached the MII stage when vitrification was performed at or before MI compared to 85% in control (non-vitrified group in Experiment 1). However, a greater proportion of oocytes progressed from MI to MII if vitrification occurred at MI rather than at GV or GVBD. Most oocytes vitrified at the GV stage (0 h) proceeded to GVBD and MI upon resumption of IVM. Comparable cleavage rates in COCs vitrified after 0, 12 or 22 h of IVM indicate that survival is similar at different meiotic stages. The estimated cleavage rate of surviving COCs after vitrification (60%; 15% oocytes cleaved/25% oocytes developing to MII) was similar to non-vitrified control (88%; 74% oocytes cleaved/85% developing to MII). When calculating the blastocyst rate as a percentage of cleaved oocytes, 2% of vitrified oocytes developed to the blastocyst stage. This indicates that fertilization failure may not be the major cause of failure of embryonic development. Rather, vitrification may affect genomic activation of cleaved embryos, i.e., embryonic failure occurred at the early stages of growth as indicated by arrested development at 2 to 8 cell stage.

The results of the first study confirmed that most oocytes were at GV stage at the time of aspiration from ovarian follicles and that they followed the expected normal kinetics of oocyte maturation during IVM. Our data on the occurrence of different nuclear stages, i.e., GV, GVBD, MI and MII, at different times during IVM are comparable to other published studies, validating our maturation protocol [106, 109].

There is a controversy in literature regarding the suitable nuclear stage for oocyte vitrification. In some reports, stage of nuclear maturation of oocytes had no effect on survival following cryopreservation [80, 106]; whereas in others GV [105, 135], GVBD [146] and MII [50, 147] were the most suitable stages for vitrification of oocytes [109]. In the present study, nuclear stage at vitrification had no effect on oocyte maturation, cleavage or blastocyst development.

In Experiment 2, the proportion of vitrified oocytes reaching MII did not differ between 0, 6 and 12 h treatment (vitrified) groups. The lower maturation rates in oocytes vitrified at 0, 6 and 12 h (Table 4.1) than in non-vitrified controls (Figure 4.1), indicate that severe cellular damage occurred during vitrification. Although the oocytes were vitrified at different nuclear stages, a proportion of vitrified oocytes reaching MII stage in all groups indicate survival and the ability to mature *in vitro*; i.e. in comparing the proportion of oocytes at MII in Experiments 1 and 2, respectively: 1 vs 23% were MII at 0 h, 4 vs 23% were MII at 6 h and 5 vs 35% were MII at 12 h. Furthermore, the percentage of oocytes that remained at the GV stage following vitrification and completion of the 22 h of IVM were lower in Experiment 2 compared to Experiment 1 respectively: 0 h (89 vs 14%) and 6 h (46 vs 3%). This indicated survival following vitrification from the ability of the vitrified oocytes to resume IVM following warming. Oocytes vitrified after 22 h IVM had a higher maturation rate (89% MII, Table 4.1), but the data in Experiment 1 indicate that 84% of oocytes had already reached the MII stage at the time of vitrification (Figure 4.1). Therefore, the survival rate of oocytes following 22 h of IVM cannot really be evaluated until cleavage following IVF was assessed.



During the progression from the GV to MII stage, oocytes undergo several changes. Alterations in the synthesis and storage of proteins and RNA, reorganization and migration of organelles and cortical granules, cytoplasmic and perivitelline space changes, as well as morphological transformations in cumulus cells are all associated with the transition from diplotene stage of the first meiotic prophase to metaphase II and the extrusion of the first polar body [109]. Consequently, they did not undergo maturation, as witnessed in Experiment 2, or become fertilized and subsequently cleave and develop to blastocysts, as witnessed in Experiment 3. Therefore it can be postulated that many of the vitrified oocytes did not undergo the changes necessary for progression from GV to MII stage, possibly due to damage of organelles during vitrification.

Nuclear stage at the time of vitrification appears to have no effect on fertilization or embryo development (Experiment 3). However, cleavage and blastocyst rates were significantly lower in the vitrified groups than in the non-vitrified control group, suggesting that developmental competence for blastocyst formation was impaired in vitrified bovine oocytes.

Spindle disorganization followed by microtubule depolymerization has been reported to be a common manifestation in vitrified MII oocytes [50, 109, 148]. Such damage can result in chromosome scattering or increased polyploidy due to incomplete second meiosis at fertilization. Therefore, the cryopreservation of immature GV oocytes has been preferred because the meiotic spindle is not organized and the genetic material is confined within the nucleus at this stage of development. However, immature, GV oocytes are thought to be more sensitive to anisotonic stress and have lower cell membrane stability than MII stage oocytes [107]. Success in the

cryopreservation of immature oocytes largely depends on the ability to preserve the structural and functional integrity of the entire oocyte and cumulus cells surrounding the oocyte. Freezing damage associated with structural injury to the membrane and destruction of gap junctions, result in disruption of intercellular communication between the oocyte and cumulus cells [50, 105, 107, 149].

Cryopreservation of an intermediate stage oocyte, such as GVBD, has been thought to avoid some of the cryoinjuries associated with cryopreservation of GV or MII oocytes [107]. A study done in 1998 examined the effect of vitrifying oocytes following 0, 6, 12 and 24 h of IVM on the survival of bovine oocytes. The authors found that vitrification of oocytes following 12 h of IVM seemed to result in higher survival rates than other stages of maturation, since the fertilization rate was the highest (36%) among the vitrification groups [105]. However, Men et al. (2002) concluded that MII stage oocytes had more resistance to cryopreservation than GVBD stage oocytes, as indicated by the higher proportion of cleaved embryos from vitrified MII oocytes that developed into blastocysts than vitrified GVBD oocytes (1 vs 8%, respectively) [50]. Regardless of the nuclear stage cryopreserved, successful fertilization also depends on cytoplasmic maturation and consequently, any damage to cytoplasmic membrane may compromise the viability of oocytes [147].

Our results revealed no differences in embryo developments among oocytes vitrified at different nuclear stages, which support the findings of others [80, 106]. The vitrified oocytes had low rates of embryo development when compared to non-vitrified controls, indicating probable damage to the cells during vitrification, regardless of the stage of nuclear maturation. Possible

adverse consequences of cryopreservation include osmotic injury, toxic effects of cryoprotectants, concentrated intracellular electrolytes, ice crystal formation and chilling which have been reported to lead to zona fracture and alterations in intracellular organelles and cytoskeleton [150]. Vitrification has also been reported to cause the premature release of cortical granules, resulting in the modification of the zona pellucida and corresponding alterations in blockage of sperm entry [149, 151]. Aneuploidy and polyploidy have been reported to be leading causes of compromised developmental competence following cryopreservation of bovine oocytes [147, 152]. Moreover, chilling can cause apoptosis, chromosomal aberrations, modifications in mitochondria and nucleoli, as well as irreversible phase changes of lipid bilayers and membrane lysis [148, 153].

Following warming in the present studies, vitrified COC underwent some degree of cumulus expansion; however, this was not recorded and compared to non-vitrified controls. Another observation from the present study was that cumulus cells from vitrified oocytes were easier to remove than from the non-vitrified controls, possibly due to damage to the cumulus-oocyte gap junctions which are required for nuclear and cytoplasmic meiotic competence [106]. Damage to gap junctions could result in reduced oocyte maturation and subsequent embryo development, as was observed in Experiment 3 [109].

In conclusion, nuclear maturation, fertilization and embryo development of vitrified bovine oocytes was lower than in non-vitrified control oocytes, likely due to cryodamage. Results from this study indicate that a greater proportion of oocytes transition from MI to MII if vitrification occurs at MI rather than at GV and GVBD stages. Nuclear status of vitrified oocytes had no

effect on fertilization or subsequent embryo development. Future studies are required to reduce cryo-injuries to bovine oocytes and COC, and to study the mechanisms involved in reduced embryonic development of vitrified bovine oocytes.

## **CHAPTER 5: EFFECT OF LENGTH OF EXPOSURE TO CRYOPROTECTANT AND WARMING SOLUTIONS, AND VITRIFICATION OF BOVINE CUMULUS OOCYTE COMPLEXES ON CLEAVAGE AND SUBSEQUENT EMBRYO DEVELOPMENT**

### ***5.1 Abstract***

The present studies aimed to evaluate the effect of time interval of exposure of bovine cumulus oocyte complexes (COCs) to cryoprotectant and warming solutions, and vitrification on cleavage and subsequent embryo development following *in vitro* fertilization (IVF). In Experiments 1 and 2, COCs were exposed to vitrification solution 1 [VS1; TCM-199 + 7.5% ethylene glycol (EG) + 7.5% dimethyl sulfoxide (DMSO) + 20% calf serum (CS)] for 5 min (Group 1), VS1 followed by vitrification solution 2 (VS2; TCM-199 + 15% EG + 15% DMSO + 0.5 M sucrose + 20% CS) for 1 min (Group 2) and VS1 and VS2 followed by vitrification in liquid nitrogen using cryotop method and 1 min in warming solution (TCM-199 + 0.5 M sucrose + 20% CS), (Group 3). In Experiment 2, COCs in VS1, VS1+VS2 and vitrified groups were exposed to the warming solution for either 1 or 5 min. In both experiments, all COCs underwent *in vitro* maturation (IVM), IVF and *in vitro* culture (IVC). Untreated (non-vitrified) COCs underwent IVM, IVF and IVC as controls. Cleavage and blastocyst rates (as a percentage of cleaved oocytes) in the vitrified group (41 and 4% in Experiment 1, and 25 and 7% in Experiment 2, respectively) were lower ( $P<0.001$ ) than in the other groups (non-vitrified controls, 92 and 37% in Experiment 1 and 75 and 36% in Experiment 2; VS1 group, 79 and 32% in Experiment 1 and 68 and 29% in Experiment 2; VS1+VS2 group, 80 and 26% in Experiment 1 and 63 and 36% in Experiment 2). Cleavage and blastocyst rates did not differ between control, VS1 and VS1+VS2 groups. In Experiment 2, warming time did not affect cleavage rates. In conclusion, the cryoprotectants

(EG and DMSO) present in VS1 and VS2 had no adverse effects on cleavage and blastocyst rates of bovine COCs. However, vitrification of bovine COCs drastically reduced cleavage and blastocyst rates.

## ***5.2 Introduction***

Developing a reliable method for cryopreservation of oocytes would have numerous applications for assisted conception in humans, animal breeding programs, and conservation of female animal genetic resources. Oocyte cryopreservation has been performed in several mammalian species; however, the success rate is extremely low. Conventional slow freezing is commonly used for cryopreservation of embryos; however, cellular damage often results from intracellular ice formation [48]. Therefore, vitrification is being used increasingly for cryopreserving tissue, embryos and oocytes to avoid chilling injury [135, 154, 155]. It does not require expensive coolers, special skill and is a fairly quick procedure.

Vitrification uses ultra-rapid cooling and warming rates to pass the oocyte through critical temperature zones quickly [48]. It results in solid-glass formation in the oocyte and surrounding solution, due to the increased viscosity from the high concentration of cryoprotectants and rapid cooling [156]. Although successful vitrification has been reported in mouse oocytes [145], vitrification of bovine oocytes is challenging due to their complex structure and sensitivity to chilling [120]. Studies in our lab examined the effect of vitrification of bovine oocytes on nuclear maturation, cleavage and blastocyst development (Chapter 4). We concluded that

compared to non-vitrified oocytes, those which were vitrified had reduced embryo developmental competence, as indicated by low blastocyst rates (<5%).

The main biophysical factor causing cellular disruption during cryopreservation is intracellular ice crystal formation; however, this may be reduced or diminished by increasing the viscosity of the solution by using proper cryoprotectants at higher concentrations [48, 109]. The most commonly used permeating cryoprotectants for oocyte cryopreservation are ethylene glycol (EG), propylene glycol, glycerol and dimethyl sulfoxide (DMSO) [157]. Despite the critical importance of cryoprotectants for avoiding ice crystal formation in oocytes, the higher concentrations of cryoprotectants required for vitrification may be toxic and can result in osmotic injury to the cell, leading to reduced developmental competence [156, 158]. To investigate the low success rate after vitrification, it is important to examine the individual components of the system. Therefore, we wanted to determine the role of cryoprotectant solutions in the observed reduced rates of cleavage of vitrified bovine oocytes and subsequent embryo development.

During warming, it is essential to remove cryoprotectants and rehydrate the oocyte [159]. Oocyte swelling during warming (dilution) is an important determining factor for survival as volume fluctuations affect the plasma membrane integrity and cytoskeletal organization [160]. The proper warming time for vitrified oocytes is still uncertain. If the time in the warming solution is insufficient, cryoprotectants may not be completely removed and when directly exposed to an isotonic solution, water diffuses into the cell faster than the cryoprotectants flow out and osmotic swelling can occur beyond the volume limits of the oocyte [95]. Although oocytes can withstand a certain amount of swelling, if the volume fluctuation goes beyond the

critical volume, irreversible membrane damage can occur. Therefore, the present studies aimed to investigate the effect of the time intervals of exposure of bovine cumulus oocyte complexes (COCs) to cryoprotectant and warming solutions and vitrification on cleavage and subsequent embryo development following *in vitro* fertilization. We hypothesized that 1) the higher concentrations of cryoprotectant solutions used in vitrification procedures would have toxic effects on bovine COCs, resulting in poor cleavage and embryonic development and 2) warming vitrified COCs for a longer interval would improve the removal of intracellular cryoprotectants resulting in higher cleavage and blastocyst production rates.

### ***5.3 Materials and methods***

#### ***5.3.1 Chemicals and supplies***

Dulbecco's phosphate buffered saline (DPBS), Ca-Mg free DPBS, newborn calf serum (CS), TCM-199 and MEM non-essential amino acids were purchased from Invitrogen Inc. (Burlington, ON, Canada). Lutropin (LH) and Folltropin (FSH) were supplied by Bioniche Animal Health, Inc (Belleville, ON, Canada). All other chemicals and reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada), unless otherwise stated.

#### ***5.3.2 COCs collection and initial processing***

Bovine ovaries were collected from a slaughterhouse and transported to the laboratory at approximately 25 °C within 8 h. Ovaries were washed in normal saline and extra ovarian tissues



were removed. Follicles <8mm in diameter were aspirated using a 18-gauge needle attached to a 5 mL syringe containing approximately 1.0 mL of wash medium consisting of DPBS supplemented with 5% CS (v/v). The aspirated follicular fluid was pooled in 50 mL conical tubes and allowed to settle. The COCs were searched under a stereomicroscope, washed (3x) in DPBS + 5% CS and COCs with more than three layers of cumulus cells and uniform cytoplasm (Grade One) were selected for further processing [136].

### ***5.3.3 Vitrification and warming procedures***

The COCs were vitrified by equilibrating in vitrification solution 1 (VS1; TCM-199 + 7.5% ethylene glycol (EG; v/v) + 7.5% dimethyl sulfoxide (DMSO; v/v) + 20% CS) for 10 min at 37 °C. After equilibration, COCs were transferred through three 20 µL-drops of vitrification solution 2 [VS2; TCM-199 + 15% EG+ 15% DMSO + 20% CS + 17.1% sucrose (w/v)] at 37 °C for a total exposure time of < 1 min. Five COCs were loaded on a cryotop (Kitazato Supply Co., Fujinomiya, Japan), under stereomicroscope and immediately plunged in liquid nitrogen (LN<sub>2</sub>). The COCs were warmed by immersing the cryotop in a 30 mm petri dish containing warming solution (TCM-199 + 20% CS and 17.1% sucrose) at 37 °C for 1 min. The COCs were then washed (3x) in wash medium at 37 °C.

### ***5.3.4 In vitro maturation fertilization and culture***

The COCs were washed (3x) in maturation media (TCM-199 supplemented with 5% CS, 5 µg/mL LH, 0.5 µg/mL FSH and 0.05 µg/mL gentamicin). For IVM, groups of 20 COCs were

placed in 100  $\mu$ L droplets of maturation media, under mineral oil and incubated for 22 h at 38.5 °C, 5% CO<sub>2</sub> in air and high humidity. For *in vitro* fertilization (IVF), frozen-warmed pooled semen from several bulls was washed through Percoll gradient (45% and 90%) [137]. After washing, sperm were added to Brackett-Oliphant (BO) fertilization media [138] to a final concentration  $3 \times 10^6$ /mL. Following 22 h of IVM, groups of 20 oocytes were washed (3x) in BO supplemented with 10% BSA (w/v) and added to 100  $\mu$ L droplets of sperm in BO, under mineral oil. After 18 h of co-incubation with sperm at 38.5 °C, 5% CO<sub>2</sub> in air and high humidity, cumulus cells and sperm attached to oocytes were mechanically removed via pipetting. The presumptive zygotes were washed (3x) through *in vitro* culture (IVC) medium CR1aa with 5% CS, 2% BME essential amino acids (v/v), 1% MEM nonessential amino acids (v/v), 1% L-Glutamic acid (v/v), 0.3% BSA and 0.05  $\mu$ g/mL gentamicin, transferred in 100 $\mu$ L IVC droplets under mineral oil and incubated at 38.5 °C under 5% CO<sub>2</sub>, 90% N<sub>2</sub>, 5% O<sub>2</sub> and high humidity. After 48 h in culture, cleavage rate was recorded and embryo culture was continued in the same drops. Blastocyst rate was recorded on Days 7, 8 and 9 (Day 0= day of IVF).

### ***5.3.5 Experimental design***

#### ***5.3.5.1 Experiment 1: Effect of cryoprotectant exposure and vitrification on cleavage and embryo development***

The COCs were divided into the following four groups: 1) control group: COCs were held in wash medium 2) VS1 group: COCs were exposed to VS1 for 5 min then kept in wash medium; 3) VS1+VS2 group: COCs were exposed to VS1 for 5 min followed by VS2 for <1 min, and

were then kept in wash medium; 4) vitrified group: COCs were exposed to VS1 for 5 min and VS2 for <1 min, vitrified in LN<sub>2</sub> using cryotop method, warmed at 37°C in warming solution for 1 min and transferred to wash medium. After treatments, all COCs underwent *in vitro* maturation, fertilization and embryo culture, as described above. This experiment was replicated five times on different dates.

#### ***5.3.5.2 Experiment 2: Effect of length of exposure to cryoprotectant and warming solutions, and vitrification of bovine COCs on cleavage and embryo development***

As in Experiment 1, COCs were randomly divided into VS1, VS1+VS2 and vitrified groups; however, the COCs in VS1, VS1+VS2 and vitrified groups were sub-divided and exposed to warming solution for either 1 or 5 min, followed by wash medium. The COCs which were not exposed to cryoprotectants served as controls. All COCs then underwent *in vitro* maturation, fertilization and embryo culture. This experiment was replicated five times on different dates.

#### ***5.3.6 Statistical analyses***

Data were analyzed using Proc Glimmix in SAS® Enterprise Guide 4.2 [141]. Analyses were performed using randomized complete block design modeling binary distribution (for yes/no response variable) for cleavage and blastocyst rates. Main effects and interactions were considered statistically different at a P-value <0.05. The effect of control, VS1, VS1+VS2 and vitrification were examined for cleavage and blastocyst rates in Experiment 1. In Experiment 2, analyses were performed using 2x2 factorial randomized complete block design. Replicate

number (1 to 5), treatment (1=control, 2=VS1, 3= VS1+VS2, 4=vitrified), warming time (1=1 min warming time, 2=5 min warming time) and binomial response (1=cleavage, 2=no cleavage) were recorded for each oocyte. Syntax of SAS program was: Proc glimmix method=quad; class replicate treatment warming time; model cleavage (event="1") =treatment|warming time /dist=bin link=logit; random intercept/ subject=replicate; run; If the P-value for treatment\*warming time interaction term from Type III sum of squares was <0.05, then lsmeans treatment warming time/ diff lines ilink or adjust= tukey; was added to the syntax for separation of group means.

## **5.4 Results**

### ***5.4.1 Experiment 1: Effect of cryoprotectant exposure and vitrification on cleavage and embryo development***

Data on effect of exposure to cryoprotectant solutions and vitrification on oocyte cleavage and embryo development are presented in Table 5.1. The cleavage rate of oocytes from control group (92%), (not exposed to cryoprotectants or cooling), did not differ from the VS1 (79%) or VS1+VS2 (80%) groups ( $P>0.05$ ). However, the cleavage rate from the vitrified group (41%) was lower ( $P<0.001$ ) than control, VS1 and VS1+VS2 groups. The blastocyst rate in the control group (34%) did not differ ( $P>0.05$ ) from the VS1 (25%) or VS1+VS2 (21%) groups, but all differed ( $P<0.001$ ) from the vitrified group (2%).

**Table 5.1** Effect of cryoprotectant exposure and vitrification on cleavage of bovine oocytes and subsequent embryo development.

	Oocytes (n)	Cleavage rate n (%)	Blastocyst rate n (% of total n)	Blastocyst rate (% of cleaved)
Non-exposed (control)	90	83 (92%)	31 (34%)	37%
VS1	107	85 (79%)	27 (25%)	32%
VS1+VS2	96	77 (80%)	20 (21%)	26%
Vitrified	127	52 (41%)	2 (2%)	4%
<i>P-value</i>				
<sup>a</sup> Cryoprotectant exposure		0.847	0.456	
<sup>b</sup> Vitrification		<0.001	<0.001	

<sup>a</sup>Analysis excluded vitrified group

<sup>b</sup>Analysis of all groups

VS1: Vitrification solution 1

VS2: Vitrification solution 2

#### ***5.4.2 Experiment 2: Effect of length of exposure to cryoprotectant and warming solutions, and vitrification of bovine COCs on cleavage and embryo development***

Data on effect of time in cryoprotectant and warming solutions and vitrification on cleavage and blastocyst rates are presented in Table 5.2. There was no effect ( $P>0.05$ ) of time in the warming solution on cleavage and embryo development. Both cleavage and blastocyst rates in the vitrified group (25 and 0.6%, respectively) were lower ( $P<0.001$ ) than in non-vitrified control (75 and 27%, respectively), VS1 (68 and 20%, respectively) or VS1+VS2 (63 and 23%, respectively) groups, which did not differ from one another ( $P>0.05$ ).

### ***5.5 Discussion***

In the present study, the effects of length of exposure to vitrification and warming solutions and vitrification on *in vitro* fertilization and subsequent embryo development of bovine COCs was examined. Results indicated that exposure of immature bovine COCs to cryoprotectants commonly used in vitrification procedures had no adverse effect on cleavage or embryo development. However, embryonic development was compromised by the vitrification procedure, suggesting that cooling is causing cellular damage. Furthermore, this study indicated that a period of 5 min in warming solution did not result in additional increase in oocyte viability, when compared to 1 min.

Vitrification requires very high concentrations of cryoprotectants to reduce the probability of intracellular ice crystal formation. However, there is a biological limit to the ability of oocytes to

**Table 5.2** Effect of time in cryoprotectant and warming solutions on cleavage of bovine oocytes and subsequent embryo development

	Oocytes (n)	Cleavage rate n (%)	Blastocyst rate n (% of total n)	Blastocyst rate % of cleaved
Non-exposed (control)	81	61 (75%)	22 (27%)	36%
VS1				
1 min warming interval	82	60 (73%)	16 (20%)	27%
5 min warming interval	77	48 (62%)	15 (20%)	31%
VS1+VS2				
1 min warming interval	87	52 (60%)	16 (18%)	31%
5 min warming interval	82	54 (66%)	22 (27%)	41%
Vitrified				
1 min warming interval	74	22 (30%)	1 (1%)	5%
5 min warming interval	98	21 (21%)	2 (2%)	10%
<i>P-value</i>				
<sup>a</sup> Cryoprotectant exposure		0.202	0.205	
<sup>b</sup> Warming time		0.241	0.689	
<sup>b</sup> Cryoprotectant exposure * warming time		0.381	0.687	
<sup>c</sup> Vitrification		<0.001	<0.001	

<sup>a</sup>Analysis excludes vitrified groups

<sup>b</sup>Analysis excludes control

<sup>c</sup>Analysis of all groups

VS1: Vitrification solution 1

VS2: Vitrification solution 2

tolerate the osmotic stress associated with high concentrations of cryoprotectants [84]. Our findings suggest that it was the ultra-rapid cooling of bovine oocytes during vitrification that adversely affected cleavage and embryo development, rather than the high concentrations of cryoprotectants (EG and DMSO). In both Experiments 1 and 2, exposure to VS1 and VS1+VS2 did not adversely affect cleavage and blastocyst rates. However, vitrification drastically reduced cleavage and embryo development rates, a consequence of the sensitivity of bovine COCs to cooling, as has been suggested by others [158].

Recently, there has been much debate about the use of DMSO in vitrification solutions on the Embryo Mail Website [161]. Oocyte vitrification using DMSO alone [162] or in combination with other cryoprotectants [112] has been reported to be successful. However, contradictory findings report the use of DMSO (alone or in combination with EG) can reduce the developmental competence of oocytes [156, 158]. Although DMSO-free vitrification systems have shown promising results with embryos [163, 164], results have not been as favorable for oocytes [165]. The ideal cryoprotectant solution has high permeability and low toxicity, but there has been no such optimal cryoprotectant combination described to date.

Several reports have shown that cryoprotective agents interfere with tubulin and filamentous actin structures of the cytoskeleton of oocytes [159, 166, 167]. Moreover, cryoprotectants have been thought to cause premature cortical granule release resulting in zona hardening and reduced subsequent fertilization rates [7, 159, 168]. In the present studies, COCs exposed to cryoprotectants (without vitrification) had satisfactory cleavage and blastocyst rates suggesting that sperm penetration was apparently not an issue. In this regard, CS has been reported to be



protective against zona hardening and defective blocking of polyspermy in the mouse [169]. In the present studies, the CS used in the vitrification and warming solutions may have had a protective effect against osmotic and toxic effects of cryoprotectant exposure [7].

Cleavage and blastocyst development following cryoprotectant exposure did not differ from non-treated controls in the present experiments supporting a previous report in which the cryoprotectant and concentration had no effect on microtubule polymerization in matured ovine oocytes [81]. Likewise, Hochi et al (2001) found no adverse effects of vitrification solutions containing 1.5 and 3.0 M EG for 5 min each followed by 1 min in 7.2 M EG and 1.0 M sucrose on morphological survival, sperm penetration and fertilization of bovine oocytes [170]. Moreover, DMSO did not result in damage to microorganelles related to normal oocyte morphology and sperm penetration [171].

Bovine oocytes have been cryopreserved at different nuclear stages (germinal vesicle, germinal vesicle breakdown, metaphase I and metaphase II). However, mature oocytes have meiotic spindles that are extremely sensitive to cooling and exposure to cryoprotectants, which often results in tubulin depolymerization [89, 156, 171]. The presence of cumulus cells on immature oocytes has been reported to be beneficial to oocytes during the addition or removal of cryoprotectants by offering a rigid structure that protects against morphological damage resulting from osmotic and toxic effects [7].

The low cleavage and blastocyst rates in the vitrified groups indicate that cooling had detrimental effects on the COCs. This may be due to intracellular ice crystallization resulting from

insufficient time for cryoprotectants to cross the cumulus layer and permeate the oocytes. It has been reported that cryopreservation of oocytes results in several ultrastructural and morphological alterations including abnormal distribution of chromosomes, microtubules and actin microfilaments [150, 172]. Additionally, damage to gap junction integrity can result in disruption of the communication between cumulus cells and oocytes [109, 147]. These morphological changes have been linked to failure in fertilization and embryo development [81, 173]. During vitrification and warming in the present studies, ultra-rapid temperature changes may have resulted in damage to the membrane and ooplasm, as a result of intracellular ice formation. Consequently, disruptions in protein synthesis and the failure of cumulus cell expansion and further meiotic development may have been responsible for the fertilization and subsequent embryo development failure [174]. Therefore, it is very important to confirm glass phase transition in the oocyte during vitrification to corroborate the absence of ice crystal formation.

In addition to the osmotic stress resulting from exposure to high concentrations of cryoprotectant solutions, oocytes may also undergo dramatic volume changes during warming. During this process, oocytes are rehydrated while cryoprotectants and water are exchanged across the concentration gradient [159]. However, one of the most important factors affecting cell survival during dilution is excessive cell swelling. Water moves through the membrane faster than cryoprotectants and because of the high concentration of cryoprotectants remaining in the cell, water is drawn in and swelling occurs. It has been shown that volume fluctuations can affect the integrity of the plasma membrane as well as the cytoskeletal organization of oocytes [160]. Exposure to a warming solution is important for the removal of penetrating cryoprotectants from

oocytes; however, the ideal time is unknown. In Experiment 2, exposing oocytes to the warming solution for 1 vs 5 min intervals appeared to have no obvious differences on subsequent developmental competence, indicating that longer warming solution exposure may not have added benefits. However, 1 min may not be ideal, as indicated by the low blastocyst rates and further investigation into the optimal warming interval is required.

The cleavage and blastocyst rates in all groups in Experiment 2 were noticeably lower than in Experiment 1. This could be attributed to changes in the slaughterhouse procedures and shipping times and methods. In Experiment 1, ovaries were shipped by courier over a distance of 220 km within 6 h of slaughter. Due to unavoidable circumstances, ovaries for Experiment 2 came from a slaughterhouse 600 km away. They had to be air-shipped, and often reached the lab more than 8 h after slaughter. The change in temperature and atmospheric pressure during shipping and exposure to x-ray security machines may have influenced cell signaling of the oocytes within the follicles of the ovaries and could account for the lower developmental rates in Experiment 2.

In conclusion, the present studies indicated that exposure of immature bovine COCs to EG and DMSO based cryoprotectant solutions does not adversely affect subsequent fertilization and embryo development; however, ultra-rapid cooling to  $-196^{\circ}\text{C}$  (vitrification) clearly did. Furthermore, 5 min exposure to warming solution had no added benefits over 1 min, with regards to cleavage rates. The vitrification procedure had a severe adverse effect on cleavage of bovine oocytes and early embryo development.

## **CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS**

Cryopreservation of mammalian oocytes has several applications. The development of oocyte banks would allow for genetic improvement, global genetic transport, food security, long term preservation, maintenance of genetic diversity and breed regeneration and rescue. Furthermore, the applications of assisted reproductive technologies (ART) in domestic animals require a large number of viable oocytes. The cryopreservation of oocytes ensures their availability for ART. However, despite promising advancements in post-thaw survival of bovine semen and embryos, there are very few reports on the successful cryopreservation of bovine oocytes. This is largely attributed to the lack of understanding of the fundamental cryobiological requirements of oocytes, especially those of cumulus oocyte complexes (COCs).

Freezing rate is one of the most important factors in a cryopreservation protocol. Currently there are two common methods for cryopreservation of mammalian COCs: conventional slow freezing and ultra-rapid freezing called “vitrification”. During slow freezing, oocytes undergo slow and partial dehydration due to the formation of extracellular ice, through an equilibrium process involving the removal of water (osmosis). Slow freezing is a common method for cryopreservation of embryos, and oocytes in species such as the mouse, cat, rabbit and humans which are not highly sensitive to cooling. However, oocytes in species such as cattle and pigs have high lipid content and low membrane permeability, and consequently are more sensitive to chilling [9]. Upon freezing, the remaining intracellular water crystallizes, often resulting in cell damage. One of the leading issues with slow freezing is prolonged exposure of COCs to cryoprotectants. It is difficult to achieve an acceptable freezing rate that avoids intracellular ice crystals, osmotic shock and the solution effect[9]. Slow freezing has also been shown to have

detrimental effects on COC metabolism. An increase in intracellular calcium has been correlated with degeneration, apoptosis and parthenogenetic activation [175]. Moreover, mass spectrometry studies have identified drastic changes in protein expression in oocytes that underwent slow freezing, that are not present in vitrified oocytes [52, 176].

Vitrification, originally developed for the cryopreservation of embryos, is a promising alternative for the cryopreservation of oocytes [38]. It solves a variety of biological problems associated with conventional freezing methods and has made cryopreservation more simple and convenient. The word vitrification is derived from the Latin word “Vitrum”, which means glass. This approach uses high concentrations of cryoprotectants and ultra-rapid freezing rates to create an ice crystal-free, solid glass-like structure. Glass transition, which prevents ice crystallization, occurs due to thermodynamics. High concentrations of cryoprotectants bind with water and dehydrate the cells for a very short time, depressing the freezing point below -120 °C, the glass phase temperature. Upon ultra-rapid freezing, the viscous vitrification solution and cells within it, turn into a glass state without any intracellular ice formation [177]. Currently, vitrification is used for cryopreservation of embryos [178], ovarian tissues [179], ova [180], and whole ovaries [181]. Live offspring have been born from vitrified-warmed oocytes from the cow [112], horse [32] and human [97]. However, the overall success rate in terms of subsequent embryonic development has not been satisfactory in most species.

Although vitrification prevents damage associated with ice crystallization, there are effects on oocyte (cellular) function, which can compromise their ability to develop normally. Osmotic injury, toxic effects of cryoprotectants, concentrated intracellular electrolytes, chilling injury,

zona pellucida fracture and alterations to intracellular organelles, cytoskeleton and cell to cell contacts are the main factors contributing to compromised oocyte development following vitrification [48]. However, the severity and degree of damage to oocytes varies depending on many factors. Cryoprotectant concentrations and exposure times, cryodevices, stage of maturation, cooling and warming rates and equilibration time in the warming solution all affect oocyte developmental competence following vitrification.

The experiments presented in this thesis were undertaken in an attempt to develop methods for the cryopreservation of bovine oocytes utilizing vitrification procedures. We examined the vitrification of bovine COCs using different equilibration times, packaging devices (cryodevices), oocyte maturation stages, cryoprotectant concentrations and equilibration, and warming intervals on nuclear maturation, and subsequent cleavage and embryo development following *in vitro* fertilization.

In order to make the procedure more efficient and convenient, we examined the effect of preparing solutions in advance and storing them below 0 °C. We hypothesized that vitrification of immature bovine oocytes in frozen-thawed vitrification solutions would result in lower cleavage rates and subsequent embryo development than when freshly prepared solutions were used. We determined that vitrification and warming solutions can be stored for extended periods of time in the freezer with no effect on subsequent cleavage rates of COCs held in those solutions. Therefore, in subsequent studies, vitrification solutions (VS1 and VS2) and the warming solution were prepared and stored in freezer (-20 °C) for convenience and to avoid batch-to-batch differences in these solutions.

Equilibrating COCs in vitrification solution 1 (VS1), which contains half of the concentrations of permeating cryoprotectants of vitrification solution 2 (VS2), is generally recommended to minimize the toxic and osmotic injuries of VS2. However, removing the step of equilibration in VS1 would save time and the vitrification procedure would become more efficient by exposing oocytes to cryoprotectant solutions for a shorter period of time. One of the objectives of this thesis was to examine the effect of equilibration on nuclear maturation, cleavage and blastocyst development. In the first set of studies presented in this thesis, we hypothesized that equilibration of bovine COCs in VS1, before vitrification, is not essential for their subsequent nuclear maturation, cleavage and blastocyst development. Although there was no beneficial effect of equilibrating COCs in VS1 on post-warm nuclear maturation following vitrification, the cleavage rate was improved following equilibration in VS1 for 5 min vs 0 min. It seems that 5 min equilibration in VS1 is an appropriate time for penetration of cryoprotectants with minimum toxicity.

Currently, the cryotop is a common device for the vitrification of mammalian embryos and oocytes. It has been designed to minimize the volume of the vitrification solution surrounding the cells in order to achieve ultra-rapid cooling. For vitrification of oocytes and embryos, the cryotop has been compared with other devices (cryoloop, open-pulled straw, electron microscope grids etc) and found to yield significantly higher survival rates than other devices [87, 99]. However, the cryotop poses biosecurity risks because cells are exposed directly to liquid nitrogen, which is potentially contaminated with infectious agents. Alternately, 0.25 mL straws can be used to avoid contamination. However, the cooling rate in straw is not as rapid as with

the cryotop due to the volume of sample to be vitrified and the thickness of straw's plastic wall. One of the objectives of this study was to compare the *in vitro* maturation of COCs vitrified in 0.25 mL straws or cryotops. We hypothesized that cryotop is a more efficient packaging system (cryodevice) than 0.25 mL straws for vitrification of bovine COCs. Following warming, more oocytes reached metaphase II (MII) stage when vitrified using cryotops, suggesting that the more rapid cooling rate would yield higher survival rates following vitrification. Consequently, the cryotop was used for all subsequent studies in this thesis. However, further research on the development of a cryodevice that provides ultra-rapid cooling with no direct exposure to liquid nitrogen is necessary.

Although the results are controversial, meiotic stage is thought to contribute to oocyte survivability following cryopreservation [50, 105-109]. Moreover, the preliminary data from our laboratory revealed that as compared to controls, vitrification of bovine COCs resulted in reduced maturation rates. Therefore, we had to determine if our lab conditions were conducive for *in vitro* maturation of bovine COCs. We hypothesized immature (GV stage) bovine oocytes would mature *in vitro* in 22 h. Nearly 90% of oocytes were GV stage at the time of aspiration from ovarian follicles and 84% reached MII stage within 22 h, following the expected kinetics of oocyte maturation in our lab. The results of this study provided substantial evidence for what nuclear stage oocytes can be expected to achieve follow different intervals of time in IVM medium in our laboratory, which was needed for subsequent studies in which oocytes were vitrified at different time intervals in IVM medium. We hypothesized that the GV stage bovine oocytes at the time of vitrification would yield higher post-warm nuclear maturation and cleavage rates and subsequent embryo development. However, there was no difference in the



proportions of oocytes reaching MII, undergoing cleavage and subsequently developing into embryos due to the stage of nuclear maturation at the time of vitrification. It seemed that regardless of the stage of nuclear development, the developmental competence of the oocytes was compromised by vitrification. Although the results of these studies indicated nuclear stage at the time of vitrification has no effect on *in vitro* maturation, cleavage and embryo development, it is unknown whether a longer interval of time in IVM (i.e. greater than 22 h) would increase the proportion of vitrified oocytes reaching MII. A relatively high proportion of the oocytes vitrified at 0 h (GV stage) and 6 h (GV and GVBD stages), reached MI by 22 h. Perhaps a longer incubation time (24-26 h) would have given those oocytes a greater opportunity to complete nuclear maturation.

Despite the critical importance of cryoprotectants for avoiding ice crystal formation, the higher concentrations of cryoprotectants required for vitrification may result in toxic and osmotic injuries to oocytes, leading to reduced developmental competence [156, 158]. During equilibration, oocytes are placed in a hypertonic solution. In order to reach equilibrium, water moves out through the cell membrane and the oocyte shrinks. Cryoprotectants move through the membrane into the cell at a rate that is much slower than the rate of water leaving the cell [182]. As a result, the intracellular solute concentrations increase drastically, which can be very toxic to oocytes. Eventually the oocyte re-expands due to influx of water following cryoprotectants. However, oocytes have limited tolerance to shrinkage and swelling and this process often causes osmotic shock, which can result in damage to the cytoskeleton. During dilution, extensive swelling can also lead to osmotic shock resulting in membrane lysis and ultimately death of the oocyte [183]. Therefore, we hypothesized that exposure of bovine COCs to cryoprotectant

solutions affects their ability to become fertilized, cleave and subsequently develop into blastocysts and that the higher concentrations of cryoprotectant solutions used in vitrification procedures have toxic effects on bovine COCs, resulting in reduced cleavage and embryonic development. To test these hypotheses, we examined the cleavage and blastocyst development rates of immature COCs exposed to VS1, VS2 and warming solutions and found no apparent negative effects of cryoprotectants, as indicated by similar cleavage and blastocyst rates between the control and cryoprotectant exposed groups. From these studies, we concluded that the cryoprotectant solutions used in these experiments were likely not causing damage responsible for low development rates following vitrification of bovine COCs. However, further studies are required to quantify cryoprotectant permeation, using nuclear magnetic resonance technology [184]. Compared to the control and cryoprotectant exposed groups, the vitrified groups had reduced cleavage and blastocyst production rates, suggesting a higher sensitivity of bovine COCs to cooling rather than cryoprotectants per se.

The low maturation, cleavage and blastocyst rates of bovine oocytes that occurred following vitrification in these studies indicate that the potential of COCs to develop normally was compromised. Cryodamage has been reported to produce a disruption in protein synthesis and a failure of cumulus cell expansion and further meiotic development, resulting in fertilization failure [174]. Cryopreserved oocytes have been reported to have several ultrastructural and morphological alterations and abnormal distribution of chromosomes, microtubules and actin microfilaments as well as disorganization of the spindle apparatus leading to risk of chromosomal loss and aneuploidy, phase change of lipid bilayers and cytoplasm membrane lysis and nuclear fragmentation [150, 172]. These changes damage gap junction integrity, resulting in

disruption of the communication between cumulus cells and the oocyte [109, 147]. Morphological changes following vitrification have been linked to failure in fertilization and embryo development [81, 173] as was observed in these studies; however, in spite of achieving acceptable cleavage rates, the exact cause of low developmental rates in the present studies is unknown. We observed embryonic developmental arrest at the 4-8 cell stage following vitrification. It is at this stage that embryos take over its own genome, so vitrification may have severely affected early embryonic genes or signal transduction pathways essentially required for their development. Genes and enzymes connected to MAP kinase, stress kinase or apoptosis pathways would be worth studying to determine the possible causes of early embryonic arrest in vitrified oocytes.

During vitrification, COCs are exposed to various chemical, mechanical and thermal stresses that could compromise their functions and may cause cell death. The unfavorable developmental rates in the present studies could be a result of the ultra-rapid temperature changes during vitrification and warming, which may have caused extensive damage to membranes and structures in the ooplasm. However, damage to COCs may have also been due to solution effects or ice crystal formation resulting from partial dehydration of oocytes and thus lack of glass phase. Confirming the specific damage incurred to COCs during vitrification would be useful for applying strategies to improve their survival rate. Although it was not confirmed in the present studies, it is very important to validate whether the solution (and COC) form a glass phase during vitrification rather than crystals in order to decrease cryoinjury. Future directions should focus on confirming the glass phase during vitrification by using strategies such as Synchrotron Beamline Technology. The Canadian Macromolecular Crystallography Facility

(CMCF) at the Canadian Light Source provides the facility to confirm the absence or presence of ice crystals using X-ray diffraction technique. When present, ice crystals exhibit a powder diffraction pattern, and in the glass phase there is no powder diffraction pattern. The use of this technology would be invaluable to develop more suitable solutions for the vitrification of bovine COCs.

During warming, it is essential to remove cryoprotectants and rehydrate the oocyte [159]. Oocyte swelling during warming (dilution) is an important factor that could affect survival; volume fluctuations (and especially swelling) are not well tolerated and can affect the plasma membrane integrity and cytoskeletal organization [160]. However, the most appropriate warming time for rehydration of vitrified COCs is still unclear, depending on the cryoprotectants used and their concentration, the constituents and temperature of the warming solution. Therefore, one of our objectives was to study the effect of time of exposure of bovine COCs to warming solutions (1 vs 5 min) on cleavage and subsequent embryo development. Although we hypothesized that warming vitrified COCs for a longer interval would presumably improve the removal of intracellular cryoprotectants resulting in higher cleavage and blastocyst production rates, our results indicated no difference between 1 and 5 min warming time. This led us to conclude that a longer interval of exposure to the warming solution (5 min) was unnecessary and consequently, the procedure could be made more efficient. However, in light of the low blastocyst rates, 1 min may not be optimal and further studies examining the interval of time in warming solutions are recommended.

From these studies, we found that vitrification adversely affected the ability of bovine oocytes to undergo *in vitro* maturation, *in vitro* fertilization and subsequent embryo development. Cryotop was a superior cryodevice for vitrification of bovine COCs when compared with the conventional 0.25 mL straw and equilibration of bovine COCs in VS1 was not essential for their *in vitro* nuclear maturation, but appeared to be important for the fertilization process. Additionally, cryoprotectant exposure and the nuclear stage of oocytes at the time of vitrification appeared to have no effect on cleavage and embryo development, and there was no difference between 1 and 5 min warming time interval. The results of these studies are pertinent for the future use of bovine oocyte cryopreservation, which despite recent advancements, is not yet a well established procedure. The techniques used in these studies can be considered important steps in the development of a feasible approach for long term storage of female animal genetics.

In order to achieve successful vitrification of bovine oocytes, an optimal balance between minimizing toxic and osmotic injury and maximizing the benefits of cryoprotectants needs to be elucidated. Although considerable efforts have been made to produce mathematical models and sophisticated equipment, as well as to analyze physical, chemical and biological factors, there has been minimal success [185]. Further insight into the volumetric responses of matured oocytes to changes in osmolarity during preparation for cooling is necessary before the toxic and osmotic effects of cryoprotectants on COCs can be countered or mitigated. There is also a need for additional studies to understand the mechanisms involved in cytoskeleton and cell membrane damage during bovine COCs vitrification in order to improve post-thaw cleavage and blastocyst rates. Furthermore, continued investigation into the importance of structural/functional relationships to normal gene expression, protein translation, intracellular trafficking, epigenetic

modifications and cell development will help to better understand developmental consequences resulting from cryopreservation of COCs [52].

Future directions of bovine COC cryopreservation research should include studying alterations of the lipid membrane composition in order to improve chilling resistance. Avenues aimed at increasing cooling and warming rates in a practical fashion in order to pass COCs through the critical temperature zone quickly should continue to be examined. Although liquid nitrogen-mediated disease transmission is a rare event, it is an area of concern that needs more attention. Finding more practical means for reducing disease risk is critical before widespread application of oocyte banking occurs. Currently there are no stringent criteria to assess COC quality, other than number of layers of cumulus cells and cytoplasm morphology. Research finding evaluation markers, such as gene expression in the cumulus cells correlated with developmental rates could be used as a marker for COC quality before freezing, helping to select the COCs most suitable for cryopreservation. Although nuclear maturation following vitrification was examined in this thesis, confirmation of cytoplasmic maturation following vitrification of COCs is also essential.

The studies presented in this thesis are merely stepping stones in the pursuit of successful long term storage of mammalian oocytes. The future of this research field holds great challenges, but also great promise.

“Every great improvement has come after repeated failures. Virtually nothing comes out right the first time. Failures, repeated failures, are finger posts on the road to achievement. One fails forward toward success.” -Charles F. Kettering

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## APPENDICES

### A. Culture mediums and reagents

Name	Manufacturer	Catalog No.	Amount
7X Laboratory Detergent (ICN7667094)	MP Biomedicals	76-670-94	1 Gallon
Albumin, Bovine (crystallized and lyophilized)	SIGMA	A4378	25 g
Albumin, Bovine (Fatty Acid Free)	SIGMA	A-6003	10 g
BME Amino Acids Solution	SIGMA	B6766	100 mL
Calcium Chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ )	SIGMA	C-7902	500 g
Calf Serum	GIBCO	26010-074	500 mL
Dulbecco's Phosphate Buffered Saline (+Ca/Mg)	GIBCO	14040	500 mL
FSH Folltropin	Bioniche	867357	
Gentamicin sulfate	SIGMA	G-1264	1 g
L-Glutamic Acid	SIGMA	G-8540	
Heparin	SIGMA	H-3149	10000 unit
HEPES	SIGMA	H-6147	25 g
Hypotaurine	SIGMA	H-1384	100 mg
(+)-Lactic Acid (Hemicalucim salt: Hydrate)	SIGMA	L-4388	50 g
LH	Bioniche	E092A	
Magnesium Chloride hexahydrate ( $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ )	SIGMA	M-2393	100 g
Medium 199	GIBCO	12340-030	500 mL
MEM non-essential amino acid solution	GIBCO/Invitrogen	11140-050	100 mL
Mineral Oil	Aldrich	161403-1L	1000 mL
Percoll	SIGMA	P-1644	500 mL
Phenol Red	SIGMA	P-0290	100 mL
Potassium Chloride (KCl)	SIGMA	P-5405	50
Sodium Bicarbonate ( $\text{NaHCO}_3$ )	SIGMA	S-4019	500 g
Sodium Chloride (NaCl)	SIGMA	S-5886	1 kg
Sodium Dihydrogen Phosphate Dihydrate	FLUKA	71505	1 kg
Sodium Pyruvate	SIGMA	P4562-25G	
Water	SIGMA	W-1503	500 mL
Saline			500-1000 mL
EG	SIGMA		
DMSO	SIGMA		
Sucrose	SIGMA		

## B. Equipment and materials

<b>Equipment</b>	<b>Manufacturer</b>	<b>Cat No.</b>
Disposable centrifuge tubes	VWR	21008-929
Absorbent bench underpads	VWR	56617-018
Millex GP filter (0.22 um)	Millipore	SLGP033RS
Petri Dish 35mm	VWR	25382-064
Petri Dish 60mm	VWR	CA25382-100
Petri Dishes 100 mm	Fisher	08-757-12
Spatulas	VWR	57952-107
Weigh Paper	VWR	12578-121
100 mL Graduated Cylinders	VWR	89001-100
250 mL Graduated Cylinder		
500 mL Graduated Cylinder		
Pipette Tips (all sizes)	mjs biolynx	
.1-10 µL	mjs biolynx	US 11103700
1-200 µL	mjs biolynx	US 11110700
100-1000 µL	mjs biolynx	US 11112720
Parafilm		
20 mL syringes	VWR	53548-008
Tissue Flasks (50 mL)	VWR	CA29184-801
Glass Bottles (100 mL)	VWR	KT321000-0100
Pasteur Pipettes	VWR	CA14672-380
50 mL Beakers		
100 mL Beakers	VWR	89003-784
250 mL Beakers	VWR	89003-788
500 mL Beaker		
Falcon Tubes (15 mL)	VWR	21008-918
Falcon Tubes (50 mL)	VWR	21008-951
Centrifuge		
Water Aspirator		
Hemocytometer (Neubauer Chamber)		
Weigh Scale		
1000-5000 µL Pipette		
1000-5000 µL Pipette Tips	Biolynx	1053-0710
Bunsen Burner		
Large Filters (500 mL)	VWR	28199-585
Large Filters (250 mL)	VWR	28199-677
Eppendorf Tubes (1.5 mL)	VWR	89000-028
Vortex		
Broken Glass Container	Fisherbrand	12-009-7A